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γίγονται δὲ τῶν ἵππων αἱ μὲν καὶ ἄτεκνοι
ὄλως, αἱ δὲ συλλαμβάνουσι μὲν, οὐ δύναται
δ' ἐκφέρειν.

*Some mares are completely sterile; some
conceive, but cannot complete their
pregnancy:...*

*Aristotle
Historia Animalium, VI. xxii*

AN INVESTIGATION OF MOSAIC/CHIMAERISM
IN THE DOMESTIC HORSE (Equus caballus caballus)

A thesis presented in partial
fulfilment of the requirements for the degree of
Doctor of Philosophy in
Cytogenetics at
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ABSTRACT

This study involved three aspects of the field of Cytogenetics applied to the domestic horse (E. caballus caballus). The first aspect, the identification and investigation of individuals with cytogenetic anomalies, is fundamental to Cytogenetics. The individuals discovered, however, pointed to the close interrelationship of Cytogenetic and Genetic research. Thirdly, questions of fertility, and in one mare the tentative demonstration of an application of cytogenetics in veterinary clinical evaluation, were concerned with functions of Clinical Cytogenetics.

The primary hypothesis of this study was that individuals with abnormal sex chromosome constitutions, the majority of which are either mosaics or chimaeras, form a significant proportion of some E. c. caballus breeds, in particular the Thoroughbred Breed. Subsidiary to that hypothesis was that these mosaic/chimaeras as a group were disproportionately responsible for the sub-fertility of some horse populations, through infertility, sub-fertility and generating abnormal offspring.

Five horses were investigated. One, a XY/XYY/?XO mosaic/chimaeric stallion was fully fertile. However, he sired an XO still-born filly. Limited studies of three of his relatives and three of his other progeny, suggested the possibility of cytogenetic anomalies in that kinship. He was the first reported stallion with an XYY line, and his XO filly was the first reported still-born foal with a chromosome abnormality.

Two 64,XX/65,XXY mares with no physical signs of intersexuality were studied. One was infertile, however, there was insufficient information to assess the other mare's fertility. An infertile mare was discovered and investigated in depth, which had 65,XXX/65,XXY/64,XX/66,XXY/66,XXXX mixoploid mosaic/chimaerism, although she was primarily a triple-X mare. The fourth mare was primarily thought to

be a proliferative mosaic, which exhibited mitotic instability. Post mortem evidence, however, confirmed that she had been cytogenetically abnormal and that she probably was a mosaic/chimaera.

Aside from one XX/XXY mare, which had been born with congenital defects, there was nothing either in the history or phenotype of these five horses to distinguish them from other Thoroughbreds. Only one of the mares was presented for analysis because of infertility, although two of the other mares might have been termed "shy breeders".

The discovery of all the major sex chromosome aneuploid conditions infrequently found in humans, XO, XXY, XYY and XXX, in the mosaic/chimaeric condition among less than 200 Thoroughbreds evaluated, suggested that chromosome anomalies are prevalent in the Thoroughbred population.

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CHAPTER I

INTRODUCTION

1.1. Infertility in the Domestic Horse (E. caballus caballus): The Aims of this Study.

It has become accepted that the horse is probably the least fecund of domestic animals (Day, 1940;1957; Bergin, 1969; Osborne, 1975). The Thoroughbred breed, in particular, is considered the least prolific, with worldwide live-foal rates ranging between 50 and 70 percent (Elliot et al., 1971; Dewes, 1973; Laing and Leech, 1975; Von Lepel, 1975), and low estimated initial conception rates of approximately 70 percent (Hutton and Meacham, 1968; Elliot et al., 1971; Osborne, 1975).

The reasons for this low fecundity are multifactoral, however, some aspects of poor fertility in individual mares are similar to those of women: some mares never breed; other mares may only breed once in a life-time, displaying similarities to precocious menopause; other mares may conceive but consistently abort; while some mares, akin to primary amenorrhoea, never show oestrus. In many women such irregularities have been found to have a high correlation with cytogenetic abnormalities. The predominant chromosome abnormality involved is believed to be suspected or confirmed mosaicism (Hamerton, 1971; McDonough et al., 1977).

On the other hand, in males of several species, chromosome abnormalities have been implicated in poor reproductive performance. An hereditary tendency for chromosome abnormalities was identified in a bull (Rieck et al., 1970, 1974; Rieck, 1973). Reduced fertility was found in a 60,XY/61,XXY/59,XO mosaic bull, which had a high rate of infertile offspring including one infertile mosaic (Lajda et al., 1976). A range of fertility has been noted in haemopoietic chimaeric bulls (Dunn et al., 1979). Autosomal aneuploid mosaicism in swine was found associated with reduced fertility in two boars and two of their chromosomally abnormal daughters (Vogt et al., 1974).

In E. caballus caballus high estimated early embryonic death (DuPlessis, 1964; Bain, 1969; Dawson, 1977) and high rates of foetal abortion of "unknown" etiology (Dimock et al., 1947; Jeffcott and Whitwell, 1973; Platt, 1973) contribute markedly to the domestic horse's poor reproduction. Research in cytogenetics has shown that the majority of conceptual loss in humans is due to chromosome abnormalities (Boué and Boué, 1973; 1974; Hassold et al., 1978). At least 80 percent of mid-term spontaneous abortuses are chromosomally abnormal (Alberman, 1973; Boué and Boué, 1974; Hassold et al., 1978; Sutherland et al., 1978; Optiz et al., 1979). A similar situation could exist in Equidae.

It has been suggested that there is a high risk of chromosomally abnormal parents generating chromosomally abnormal offspring (Dewhurst, 1978; Heritage et al., 1978). Humans with abnormalities of the sex chromosome complement who are capable of reproduction, either spontaneously or following hormone therapy, with the exception of the XYY male and XXX female, are generally thought to be either mosaics or chimaeras (Morishima and Grumbach, 1968; Záh et al., 1975; Reyes et al., 1976; Kemmann et al., 1977; Dewhurst, 1978; Gemzell and Solish, 1979).

The increasing reports of infertile XO mares (Blue, 1976; Blue et al., 1978; Bruère et al., 1978; Trommershausen-Smith et al., 1979), strongly suggested that other karyotypically abnormal individuals exist within the horse population. Therefore, it seemed reasonable to propose that, like humans, the majority of chromosomally abnormal horses could be either mosaics or chimaeras, and that the mosaic/chimaeras constitute a large "class" within the population which contributes significantly to the subfertility of that population.

One frequently finds reports of individuals, of various species, with two or more different chromosome complements. However, with the exception of the freemartin chimaeras, intensive studies of natural mosaic or chimaeric individuals are few. In part, this is due to the limitation of the numbers of tissues which may be examined from live individuals (e.g., Burns et al., 1979). In humans, abortion material has been studied extensively using cytogenetic techniques, however, abortion material seldom yields enough cells even for the diagnosis of mosaicism (e.g., Boué and Boué, 1974; Hassold et al., 1978). In addition, in humans investigations for chromosome abnormalities are rarely attempted at death (Bauld et al., 1974; Sutherland et al., 1978). Thus, the most comprehensive and definitive study of a human mosaic is that of a foetus by Klinger and Schwatzer (1962). On the other hand, Cytogeneticists working with domestic animals lack the extensive resources available to those studying humans (Basrur, 1974), and investigations of mosaic and chimaeric individuals have been limited.

The object of this study was to identify and investigate individual horses with sex chromosome mosaic/chimaerism. These mosaic or chimaeric individuals might be either sterile or fertile. The fertile individuals may contribute to the poor fertility of the domestic horse by low fecundity or by generating disproportionately more chromosomally abnormal embryos, fetuses, and offspring with impaired fertility.

1.2. Mosaicism and Chimaerism: Definitions and Possible Relation to Infertility.

1.2.1. Mosaicism, Chimaerism and "Mosaic/chimaerism": Definitions

A mosaic is an individual with cell populations of more than one genotype derived from mutational or post-zygotic events; whereas a chimaera is an individual with cell populations arising through a mixture of different zygotic genotypes (Benirschke, 1971; Hamerton, 1971). It follows that chimaerism in an individual can be proved only by genetic evidence and in the absence of genetic markers it cannot be proved that an individual is not a mosaic (Ford, 1969; Fitzgerald et al., 1979).

Since chromosome analysis differentiates cell types, but does not always distinguish between chimaerism and mosaicism (Ford, 1969; Basrur et al., 1970), "mosaic/chimaerism" has been used throughout this Thesis to denote the presence of more than one cell line. A distinction between mosaicism and chimaerism in most cases has been made only in discussion and speculation as to the origins of the cell lines. However, aspects of chimaerism and mosaicism may differ in their influences on fertility.

1.2.2. Chimaerism: Types, Possible Relation to Fertility and Possible Predisposing Factors.

There are two general types of chimaerism relevant to fertility: chimaeras of the endo-reticular system, or "haemopoietic" chimaeras, and true chimaeras, or "whole body" chimaeras.

1.2.2.1. Haemopoietic chimaerism

Haemopoietic chimaerism arises from placental vascular anastomosis between twin foetuses. It is associated with the sterile freemartin intersex condition in cattle, sheep and goats (Benirschke, 1971; Jost et al., 1972, 1975), and may be implicated in the impaired fertility of some heterosexual bull twins (Dunn et al., 1979; Long, 1979).

Placental vascular anastomosis occurs in many species (Benirschke, 1972), however, the freemartin intersex condition is commonly found in cattle (Lillie, 1917; Benirschke, 1971; Jost et al., 1972). The sterile freemartin is a physically modified genetic female, whose non-haemopoietic tissues contain the normal XX sex chromosome constitution although its haemopoietic tissues may contain any proportion of XY cells (Bruère and McNab, 1968; Benirschke, 1971). Similarly, in heterosexual bull twins, which are usually fertile, XX cells have not been identified in either somatic or germinal tissues (Dunn et al., 1979).

In the domestic horse chorio-vascular anastomosis is thought to occur in approximately 50 percent of twin gestations (Bouters and Vandeplassche, 1972; Spincemaille et al., 1975). However, the freemartin condition has not been identified in Equidae (Basrur et al., 1970; Vandeplassche et al., 1970; Spincemaille et al., 1975).

1.2.2.2. Whole body chimaerism

In a whole body chimaera there is a mixture of genotypes distributed throughout the body (Ford, 1969; Benirschke, 1971; Hamerton, 1971). These true chimaeras can develop from one ovum following dispermic fertilization, alternatively, they can arise from the fusion of two fertilized ova.

Dispermy was thought to have occurred in humans (Gartler *et al.*, 1962; Giblett *et al.*, 1963; Zuelzer *et al.*, 1964; Fitzgerald *et al.*, 1979); in cattle (Dunn *et al.*, 1979); and in mice (Russell and Woodial, 1966). The fusion of two fertilized ova, while considered possible, has yet to be proved (Klinger and Schwarzacher, 1962; Ford, 1969; Benirschke, 1971). The major support for the possibility of fusion has come from the frequency with which multiovular follicles are found in neonate and infant human ovaries (Bacsick, 1950). In addition, a familial chimaerism was found in a strain of mouse which prematurely produced uterine lysine (Polani, 1973).

The majority of individuals identified as whole body chimaeras have been heterosexual admixtures which had atypical sexual development or were infertile (Ford, 1969; Benirschke, 1971). In humans, such chimaeras may be hermaphrodites, normal females with gonadal dysgenesis or normal men and women (Zuelzer *et al.*, 1964; Bain and Scott, 1965; Polani, 1973; Fitzgerald *et al.*, 1979).

Aside from gross developmental anomalies that can occur in the heterosexual admixtures, there is evidence suggesting that the autosomal admixture of the chimaeric condition might predispose to poor reproductive capacity through the development of an autoimmune reaction either against one cell line or towards a specific type of cell of one line (Dunn *et al.*, 1979).

1.2.3. Mosaicism: Possible Implications in Infertility and Possible Predisposing Factors.

As well as the sex chromosome mosaic condition being a relatively frequent finding in persons examined for infertility (Kjessler, 1965; Miro *et al.*, 1978; Hsueh *et al.*, 1978), parental mosaicism has been suggested to contribute significantly to conceptual loss by fertile humans (e.g., Dewhurst, 1978; Heritage *et al.*, 1978). The report of a subfertile bull with sex chromosome mosaicism

(Lajda, 1976) and isolated reports of sex chromosome mosaicism in infertile mares (see section 1.7.2.) suggests a relationship between mosaicism and fertility could exist in most mammals.

A significant proportion of human sex chromosome abnormalities are thought to occur in the mosaic condition (Hsueh et al., 1978; Maeda et al., 1978; Burns et al., 1979). Even when the mosaic chromosome constitutions do not apparently differ, a range of gonadal and morphological expression of that condition can be found among individuals (Richards and Stewart, 1978).

However, the possible amounts of differential expression by cell lines in an individual with sex chromosome mosaicism is limited, since the autosomes are identical in all the cell lines. On one level, differences in cell cycles, thought to be influenced by differing amounts of late replicating heterochromatin (Barlow, 1972; 1973; Mittwoch and Delhanty, 1972), could directly affect tissue and organ formation. On another level, diverse functioning of specialized sex-dependent cells, for example the sex hormone sensitivity of target cells (Dörner, 1975), could indirectly influence cellular function. Thus the mere presence of sex chromosome mosaicism in the embryo might influence subsequent fertility through gross developmental abnormalities, while at the cellular level it may influence sexual development and eventually the reproductive capacity of the mature animal.

1.2.3.1. Factors which might predispose to formation of mosaicism

Some, if not all, abnormal karyotypes may be predisposed to mosaic formation. For example, a considerable number of XXY Klinefelter males are XY/XXY mosaics (Hsueh et al., 1978) and an increasing number of "XX" males have been confirmed as XX/XXY mosaics (Miro et al., 1978). Most XY/XXY mosaics are thought to have originated from XXY zygotes (Race and Sanger, 1969) and if the most simple method of formation is considered, an XXY origin could be postulated for XX/XXY mosaics (Ford, 1969).

The events leading to the formation of a zygote with an abnormal karyotype might predispose to formation of the mosaic condition in such conceptuses. That is, factors that influenced the meiotic process resulting in an aneuploid gamete, might also have had an influence on the cleavage and/or mitotic processes of the conceptus.

In humans, the frequent association of certain conditions with monosomy-X patients and their relatives, and other conditions with sex-chromatin positive Klinefelter's syndrome patients and their relatives, lead medical researchers to suggest an etiological relationship between the conditions and the cytogenetic abnormality (Nance and Uchida, 1964; Fialkow, 1966; Vallotton and Forbes, 1967; Nielsen *et al.*, 1969; Irvin *et al.*, 1970; Hsueh *et al.*, 1978).

In addition, mosaicism has been suggested to be a manifestation by structurally abnormal chromosomes towards mitotic instability (Morishima and Grumbach, 1968; Armandares *et al.*, 1977). Minor structural chromosome rearrangements have also been implicated in abnormal meiosis in some humans with poor fertility (Ford and Lester, 1978; Gustavson and Kjessler, 1978; Heritage *et al.*, 1978; Karden *et al.*, 1980). In-vitro cultures of cells from phenotypically normal persons with structural rearrangements of a chromosome, such as a peri-centric inversion, have demonstrated significant karyotype alterations and mitotic instability (Fryns *et al.*, 1978; Ford and Lester, 1978).

1.2.3.2. The formation of mosaicism

Mosaicism results from an error during any phase of either cleavage or mitotic division. Errors occurring during metaphase are probably associated either with mis-migration of the centrioles at the start of metaphase¹ or mis-alignment of the chromosomes at the metaphase plate. Both non-disjunction (failure of the two chromatids to move to opposite poles) and anaphase lag, where a chromatid is excluded from both daughter cells due to failure of spindle apparatus and chromatid interaction, are errors of Anaphase. Telophase errors are related to malfunctions of cytoplasmic systems which influence cytokinesis. Errors of chromatid replication are associated with interphase and prophase.

¹Centrioles are not normally present during the first few cleavage divisions (Szöllösi, 1973).

1.3. Factors Affecting Mosaic/Chimaeric Composition and their Interpretation

Although studies of mosaic/chimaeric individuals have been limited, the phenomena of mosaicism and chimaerism have been used extensively in experimental systems to explore aspects of cellular, developmental and reproductive biology (Mintz, 1968; Nesbitt and Gartler, 1971; Lyon, 1972; Mintz, 1974; Tucker et al., 1974, 1978; Gartler and Andina, 1976).

The relative developmental stage at which mosaic/chimaerism arises; the sampling of the precursor pools by tissue primordia; selection in-vivo; differential proliferation, cell migration, admixing of germinal tissues and clonal growth; can all affect the mosaic/chimaeric composition of an individual (Ford, 1961; Gartler and Linder, 1964; Ford, 1969; Nesbitt and Gartler, 1971; Fialkow, 1973; Falconer and Avery, 1978).

Theories of patterns of tissue morphogenesis, cell cycles, cell selection, cell lineage relationships and primordial cell pool size estimation, derived from mosaic/chimaeric experimental systems have been used to interpret an apparent mosaic/chimaeric condition in individuals (Brown and David, 1969; Nesbitt, 1971; Nesbitt and Gartler, 1971; Lyon, 1972; Barlow, 1973; Fialkow, 1973; Gartler and Andina, 1976; Falconer and Avery, 1978).

1.4. Aspects of the Cytogenetic Evaluation of Mosaic/Chimaerism

1.4.1. Chromosome Preparations from Cultured Cells in Relation to the In-Vivo Mosaic/Chimaeric Condition.

To assume the evidence of chromosome preparations from cultured cells reflects the in-vivo situation in a mosaic/chimaera one must, in the first place, postulate that the sample cultured was representative. One further assumes that all cell lines were equally affected during transfer to culture conditions, and that neither differential growth nor a difference in viability existed in-vitro between cell lines. In addition, if an in-vitro clone line has occurred it is assumed that it can be identified and like cells representing incidental (artifactual) events during culture and harvest be excluded from evaluation (Polani, 1961; Becker et al.,

1963; Hamerton, 1971; Littlefield and Mailhes, 1975; Benn, 1977b; Wahlström, 1978).

1.4.1.1. Representativeness of sample: variation in expression of a mosaic/chimaeric condition

There are many forms of expression of mosaic/chimaerism in individuals. One line may be so minor as to be virtually undetectable (Fryns et al., 1978; Wilroy et al., 1978; Genest, 1979), a line may be limited to one or two tissues (Carr et al., 1962; London et al., 1964; Basrur et al., 1969, 1970; Mittwoch and Delhanty, 1972; Bidot-Lopez et al., 1978; Wilroy et al., 1978; Burns et al., 1979) and various degrees of mosaicism can occur in various tissues (Gartler et al., 1962; Klinger and Schwarzacher, 1962; Lewis et al., 1963; London et al., 1964; Basrur et al., 1969, 1970; Dunn et al., 1970; Taylor, 1970; Dain and Bridge, 1978). The proportions of the cell lines can vary within regions or samples of the same tissue (Gartler et al., 1962; Lewis et al., 1963; Becker et al., 1963; London et al., 1964; Gemzell and Solish, 1979). In addition, morphologic or genetic evidence may suggest that a stem cell line had been lost, as in the rare cases of Turners syndrome males and human intersexes when mosaic/chimaerism was not found (de la Chapelle et al., 1964).

A sample may represent a tissue only at the time at which it was taken. Apparent shifts in the proportions of cell types present in a mosaic/chimaera may reflect sampling error, relate to physiological differences, or may truly reflect fluctuations occurring in-vivo (Gartler, 1964; Stone et al., 1964; Chaudhuri et al., 1977; Fan et al., 1977). Non-specific variation over time in proportions of abnormal cells in sex chromosome mosaics was found by Reitalu (1967) and Tennes et al. (1975). In tetraparental sheep chimaeras, no evidence of variation was reported in the proportions of phytohaemagglutinin stimulated lymphocytes, however significant variation over time was found in proportions of different red-cell types (Tucker et al., 1974, 1978). Variations in proportions of erythrocyte types was also suggested to occur in cattle haemopoietic chimaeras (Stone et al., 1964; Dunn et al., 1979). In cattle haemopoietic chimaeras sampled at various ages, however, Greene et al. (1977) found no evidence of a change in the proportions of mitotic lymphocytes. There appeared to be some variation in some animals but not in others when

similar sheep chimaeras were investigated for variation in proportions of mitotic lymphocytes (Bruère and McNab, 1968; Dain, 1974).

1.4.1.2. Differential viability and growth of cell lines in culture

Boué and Boué (1973, 1974, 1976) reported an overall slower generation time and decreased life span of aneuploid cell cultures initiated from spontaneous human abortion material. Angel (1969) and Barlow (1972, 1973) found a significant decrease in the mitotic cycle of XO cells in cultures derived from live born human aneuploid individuals. Similarly, Barlow (1972) found the same disparity in mitotic cycle duration between cell types in XO mosaic cell cultures. A scale of proliferative advantage (differential growth) was postulated in inverse proportion to the amount of heterochromatic chromosomal material present, i.e., XO-XY-XX-XXY-XXX-XXXY-XXXX (Mittwoch et al., 1969; Barlow, 1972, 1973; Mittwoch and Delhanty, 1972).

1.4.1.3. Natural, artifactual and symptomatic aneuploidy and hyperploidy

Although in both sexes age related aneuploidy was characterized by either loss or gain of sex chromosomes, there was a marked increase in age related somatic cell aneuploidy in women (Jacobs et al., 1963; Lawler and Reeves, 1976; Fitzgerald and McEwan, 1977; Galloway and Buckton, 1978). The degree of loss with age of sex chromosomes appeared to vary from tissue to tissue (Fang et al., 1975; Shoemaker, 1977). Shoemaker (1977) suggested that age related aneuploidy may only be important in those tissues with a high mitotic rate. Further, a significant increase in aneuploid cells was found in cultured cells from individuals with minor structural chromosome rearrangements (Ford and Lester, 1978; Fryns et al., 1978). An increase in aneuploid cells with age was found in ewes (Bruère, 1966); however levels of natural aneuploidy have not been determined for other domestic animals.

Polyploid cells occur normally in some tissues (Stern, 1958; Bain and Gauld, 1964; Lancet, 1964) and occur in-vivo in some pathological conditions (Freedman et al., 1964; Lancet, 1964). On the other hand, hyperploidy in cell cultures was thought to probably represent a non-specific response to a variety of conditions (Lancet, 1964; Fialkow, 1966), such as pH changes outside a very narrow range (Ford, 1973; Ingalls and Shimada, 1974). Hyperploidy can also be increased

significantly if a culture contains a structurally abnormal chromosome (Ford and Lester, 1978) and may be increased if chimaerism is present (Martin and Sprauge, 1969).

1.4.1.4. Mitogenic stimulation and mitotic inhibition: possible harvest artifacts specific to mosaic/chimaeric conditions

No evidence was found that two cell populations influence each other with respect to the duration of their respective cell cycles in-vitro (Barlow, 1972). At harvest only those cells whose susceptible mitotic phase intersects the mitotic inhibition period will be sampled (Rotenberg, 1977). Therefore, in mosaic/chimaeric cell cultures, if the cell cycles differ, and in leucocyte cultures if either the sensitivity of the lines to the mitogen differ (Younkin, 1972) or the phases of the cell lines present differ in their susceptibility to mitogenic stimulation (Chauduri et al., 1977; Fan et al., 1977), cytogenetic analysis of cultured cells may reflect this rather than the true mosaic/chimaeric composition.

1.4.2. Mosaic/Chimaerism and Sex-Chromatin

Barr and Bertram (1949) discovered that the small, stainable (chromatin) body, which cytologists had often described in the nuclei of mammalian nerve cells, was present only in females. In 1959 Ohno and colleagues demonstrated that this chromatin body was derived from a single X-chromosome, and Lyon (1961) presented the hypothesis that the Barr body was the cytological manifestation of underlying genetic inactivation.

The sex-chromatin, X-chromatin or Barr body is a characteristic mass of chromatin found within the nuclei of some interphase somatic cells of normal XX female animals (Moore, 1966a). The location of a Barr body within the nuclei can vary depending upon the tissue type, the developmental or maturational stage of the tissue or cell, specific intercellular metabolism, and the presence of abnormal physiological and pathological conditions (Bertram, 1966; Klinger, 1966; Plotnick et al., 1971).

Not all cells show a sex-chromatin body and its absence does not necessarily indicate that all X-chromosomes are not present (in a female) or that they are active (Lyon, 1972). The proportion of cells in a normal female that displayed a Barr body was found to vary both between and within species, as well as between tissues of an

individual (Barr, 1966; Lyon, 1972; Shoemaker, 1977). However, the majority of that variance was thought to be due to technical problems, for example tissue selection, poor fixation and embedding and sectioning tissues too thinly (Barr, 1966; Klinger, 1966; Lyon, 1972). The variation in the frequency of sex-chromatin bodies found in normal women, however, means that sex-chromatin studies have limited value, by themselves, as a test for low level mosaicism in humans (Lennox, 1966; Hamerton, 1971; Rary *et al.*, 1978). On the other hand, techniques developed for the identification of "Y" chromosome chromatin (Pearson *et al.*, 1970) have been successful in identifying low level "Y" chromosome mosaicism in humans (Retief *et al.*, 1975; Miro *et al.*, 1978). However, no report was found either of the use of these techniques or their application to domestic animals.

In domestic animals, particularly ruminants and horses, few tissues other than nerves can be examined for sex-chromatin because of the presence of large coarse chromatin bodies in the interphase nucleus (Moore, 1966c; Bruère and McNab, 1968). However, strong sex dysmorphism for sex-chromatin bodies is present in the neurons of domestic animals and sex-chromatin studies of neurons are a reliable indicator of genetic sex. In haemopoietic chimaeric free-martin sheep, Bruère and McNab (1968) found significantly fewer sex-chromatin bodies than in control ewes, which they considered possibly due to the influence of the chimaeric condition on metabolic processes.

In the domestic horse, sexual dysmorphism was reported in nerve cells, epithelium and smooth muscle of the duodenum, the walls of large blood vessels and cultured skin (Hoshino and Toryu, 1959; Moore, 1966c). Sex-chromatin has been reported as part of the evaluation of horses with chromosome or phenotype abnormalities as follows: oral mucosa (Basrur *et al.*, 1969; Gluhovschi *et al.*, 1970); conjunctival mucosa (Gluhovschi *et al.*, 1970); ovarian stroma and epithelium of blood vessels (McIlwraith *et al.*, 1976); gonadal tissue (Bielánski *et al.*, 1977); and neurons of the spinal cord and cerebellum (Blue, 1976; Blue *et al.*, 1978).

1.5. Atypical Development and Cytogenetic Abnormalities

The rate of growth of tissues, organs and the individual are dependent on the length of the cell cycle and the life span of the constituent cells. In individuals with chromosome anomalies, decreased rate of growth has been postulated to cause asynchronous ontogeny, disturbances in normal embryonic induction mechanisms, reduced size and reduction in the numbers of cells in various organs, growth disorganization, disturbed intra-uterine homeostasis and most human abortions (Barlow, 1973; Benirschke, 1973; Cure et al., 1973; Philippe, 1973; Poland and Miller, 1973; Boué and Boué, 1974; Polani, 1974; Golbus, 1978). In addition, studies utilizing the expression of genes on sex chromosomes raised the question of whether abnormal sex chromosome inactivation contributes to the atypical phenotypes of some XXY males, triplo-X females and possibly some XX females (Beutler, 1964; Lyon, 1972; Bühler, 1977; Kamoun et al., 1978; Wachtel, 1980).

However, in some sex chromosome aneuploid individuals and heterosexual chimaeras an abnormality in rate of growth may be manifest only in tissues involved in reproduction, such as the failure of gonadal-germ cell interactions (Singh and Carr, 1966; Kennedy et al., 1977; Luciani et al., 1977; Højager et al., 1978; Rivelis et al., 1978; DeVicтор et al., 1979; Cunha et al., 1980).

Germ cell development is possible in females with abnormal sex chromosome complements. Ova are known to exist in XO mice (Welshons and Russell, 1959; Morris, 1968; Lyon and Hawker, 1973), infant XO girls (Conen and Glass, 1963; Singh and Carr, 1966; Carr et al., 1968), a few XO women (Kammann et al., 1977; Philip and Sele, 1977; Dewhurst, 1978; Magenis and Bennett, 1978) and most triplo-X women (Dewhurst, 1978). Studies of the expression of genes located on sex chromosomes has lead to knowledge of sex chromosome activity during gametogenesis (Epstein, 1969, 1972; Gartler et al., 1973, 1975; Gartler and Andina, 1976; Bühler, 1977; Migeon and Jelalian, 1977; Burgoyne, 1978). Similar studies of aneuploid ova confirmed that these could complete the meiotic process and also suggested that an alternative (non-random) meiotic segregation mechanism preferentially formed normal gametes (Kaufman, 1972; Burgoyne and Biggers, 1976; Luthardt, 1976; Monk and Kathuria, 1977; Monk and Harper, 1978).

1.6. Family EQUIDAE

There is a relatively prolific fossil record of the family Equidae since its proposed branching from the Perissodactyl stem approximately 56 million years ago (Axe, 1907; Simpson, 1950; Willoughby, 1974). Taxonomists differ in their classification of the surviving members of the Genus Equus (Simpson, 1950; Willoughby, 1974; Klingel, 1975; Short, 1975a). In this Thesis the taxonomic classification of sub-species of E. caballus (sp) has been used for the domestic horse (E. c. caballus) and Przewalskii's horse (E. c. przewalskii) (Simpson, 1950; Willoughby, 1974), because this is consistent with karyotype similarities and the known fertility of their hybrids (Chandley et al., 1975a; Ryder et al., 1978).

1.6.1. The Karyotypes of EQUIDAE

Bush et al. (1977) calculated that Equidae exhibit the highest rate of chromosomal evolution amongst animals, since they had evolved from a common ancestor within the last four to five million years and have developed such divergent karyotypes (see Table 1-1).

Table 1-1

Extant members of the family Equidae.

CLASSIFICATION	COMMON NAME	2N	N.F.
<i>E. caballus przewalskii</i>	Przewalskii's wild horse	66	92
<i>E. caballus caballus</i>	domestic horse	64	92
<i>E. asinus asinus</i>	ass, donkey	62	102
<i>E. asinus africanus</i>	-	-	-
<i>E. asinus somalensis</i>	-	-	-
<i>E. hemionus onager</i>	onager	56	102
<i>E. hemionus kulan</i>	kulan	55,54	-
<i>E. grevyi</i>	Grevy's zebra	46	80
<i>E. burchelli boehmi</i>	Grant's zebra	44	80
<i>E. burchelli burchelli</i>	Burchell's zebra	-	-
<i>E. burchelli selouse</i>	-	-	-
<i>E. burchelli antiquorum</i>	Chapman's zebra	44	-
<i>E. zebra hartmanne</i>	Hartmann's mountain zebra	32	62
<i>E. zebra zebra</i>	Cape mountain zebra	32	-

2N = diploid number
N.F. = number of chromosome arms

(after Ryder, 1978; Ryder et al., 1978)

The dominant trend of karyotype change in Equidae has been said to be towards reduction in the number of chromosomes (Wurster and Benirschke, 1968; Ryder, 1978). Equidae's nearest relatives, the Rhinocerotidae, which exhibit the highest known diploid number of either ungulates or mammals², were believed by Wurster and Benirschke (1968) to possess a karyotype very little changed from the suggested common Perissodactyle one.

The application of differential staining techniques to members of the Equidae family has shown that centromere fusion (Robertsonian) mechanisms are alone insufficient to explain the differences in karyotypes among the species (Ryder et al., 1978). A Robertsonian mechanism is evident in the separation of the karyotypes of the sub-species E. hemionus onager and E. h. kulan (Ryder, 1978) and the sub-species E. caballus caballus and E. c. przewalskii (Chandley et al., 1975a; Short, 1975a; Ryder et al., 1978).

However, complex inter-chromosomal and intra-chromosome rearrangements separate E. asinus, E. hemionus and E. zebra hartmannae from E. caballus (sp) (Ryder et al., 1978; Ryder, 1978). Moreover, E. asinus deviates from all the Equidae by possessing an atypical submetacentric X-chromosome, which together with morphological and biochemical evidence, implied that E. asinus is physically and karyologically neither in direct lineage nor an intermediate evolutionary form of the other extant Equidae (Ryder et al., 1978).

The evidence from sub-chromosome studies of Equidae suggested that if there was a common Equidae karyotype, at some time a cytological population arose within it which gave rise to the E. caballus (sp) karyotype and that probably much later the cytological populations of the ancestral zebras and ancestral asses also developed from the common Equidae karyotype.

1.6.2. Equine Hybrids

The mammalian family Equidae is remarkable for its ability to form interspecific hybrids, even between animals with extremely

² Diceros bicornis, 2N = 84; Ceratotherium sinum and Rhinoceros unicornis, 2N = 82.

different numbers of chromosomes as shown in Table 1-2. All such hybrids, with the exception of the "intra-specific" *E. c. caballus*/*E. c. przewalskii* crosses, have been infertile (Short, 1975a,b).

Table 1-2

Equine Hybrids

	<i>E. przewalskii</i> N.F. = 92	<i>E. caballus</i> N.F. = 92	<i>E. asinus</i> N.F. = 102	<i>E. hemionus</i> N.F. = 102	<i>E. grevyi</i> N.F. = 80	<i>E. burchelli</i> N.F. = 80	<i>E. zebra</i> N.F. = 62
<i>E. przewalskii</i> 2N = 66	-	fertile 2N = 65	-	-	-	-	-
<i>E. caballus</i> 2N = 64	fertile 2N = 65		sterile "Hinny" 2N = 63	-	-	-	sterile
<i>E. asinus</i> 2N = 62	-	sterile "Mule" 2N = 63	-	-	-	sterile	sterile "Zebret" 2N = 48
<i>E. hemionus</i> 2N = 55,56	sterile	sterile	sterile 2N = 59	-	-	-	sterile
<i>E. grevyi</i> 2N = 46	-	sterile "Zebrule" 2N = 55	sterile	-	-	unknown 2N = 45	-
<i>E. burchelli</i> 2N = 44	sterile	sterile	sterile "Zebryde" 2N = 53	-	-	-	-
<i>E. zebra</i> 2N = 32	-	sterile "Zebrule"	sterile	sterile	-	-	-

Benirschke et al., 1962; Trujillo et al., 1962; King et al., 1966; Hsu and Benirschke, 1969; Gray, 1972; Hafez, 1974; Short et al., 1974; Willoughby, 1974; Chandley et al., 1975a; Hansen, 1975; Short, 1975a,b; Eldridge and Suzuki, 1976; Ryder et al., 1978.

1.7. Cytogenetics of *E. caballus caballus*

1.7.1. The Normal Karyotype of *E. c. caballus*

1.7.1.1. Chromosome identification

The chromosomes of the domestic horse have been defined by centromere index (ratio of arm length) and differential staining (banding) (Hageltorn and Gustavsson, 1974; Chandley et al., 1975b; Buckland et al., 1976; Melchior and Höhn, 1976; Ryder et al., 1978; Trommershausen-Smith et al., 1979).

Identification of individual chromosomes was at first limited to gross morphological differences such as size and relative location of the centromere. Early in the 1960's autoradiography was developed which allowed identification of the late replicating X-chromosome (Basrur, 1974) and toward the end of the 1960's all chromosomes could be individually identified by differential staining.

T. O. Caspersson and colleagues (1968) revolutionized cytogenetics with the initial discovery of differential staining (banding) techniques, which linearly differentiated chromosomes by creating transverse bands. There are numerous methods available for obtaining bands, but basically they fall into three categories. These are the original fluorescent Q-bands; Giemsa or G-bands, which are similar to Q-bands and are more commonly used; and R-bands or reverse bands, which are the reverse of Q-bands and G-bands (Lejune, 1972; Basrur, 1974; Lawler and Reeves, 1976).

In addition, Pardue and Gall (1970) developed C-band staining techniques which differentially stain "constitutive" heterochromatin of the centromere regions, regions of late replicating DNA, and secondary constrictions. C-bands have been used to identify centric fusion rearrangements and polymorphisms of the centromere region, but have contributed little to individual chromosome identification (Hageltorn and Gustavsson, 1974). However, the domestic horse does have a very distinctive C-banded X-chromosome, with a secondary dark staining band on the long arm (Blue, 1976; Buckland et al., 1976; Melchoir and Höhn, 1976).

The X-chromosome in *E. c. caballus* can usually be distinguished by its size, its two C-bands and its G-band pattern. The Y-chromosome is indistinguishable from smaller acrocentric chromosomes by size, or in some preparations by C-bands. By G-banding the

Y-chromosome has been described as staining palely with positive dark spots at the tips of the long arms (Buckland et al., 1976) and uniformly staining dark (Melchior and Höhn, 1976). In both cases the Y-chromosome was said to be approximately the size of chromosome pair number 26.

1.7.1.2. Chromosome polymorphisms

The only C-band polymorphism reported in the domestic horse was that of chromosome pair number 13, which was not found in all animals (Blue, 1976; Buckland et al., 1976). No G-band polymorphisms had been reported in the horse. However, relatively few horses have been examined closely by means of differential staining. Hatami-Monazah and Pandit (1979) reported a diploid number of 65 in six of the 17 Caspian Pony 'breed' (E. c. caballus) animals they investigated. However, they proposed on the basis of G-banding that this sub-fertile 'breed' was actually a natural hybrid population of E. c. caballus/E. c. przewalskii which exhibited chromosome polymorphism.

1.7.1.3. The karyotype of the domestic horse

Since 1912 various diploid numbers had been reported for the domestic horse (Makino, 1951), but Rothfels et al. (1959) have been generally credited with being the first to accurately report E. c. caballus's normal 2N value as 64. This figure has been confirmed repeatedly (e.g., Benirschke et al., 1962; Sasaki and Makino, 1962; Trujillo et al., 1962; Hsu and Benirschke, 1967).

The normal chromosome complement of the domestic horse consists of 31 (4 submedian, 9 metacentric and 18 acrocentric) autosome pairs and two sex chromosomes (Hsu and Benirschke, 1967; Melchior and Höhn, 1976).

While it was agreed that the X-chromosome was the second largest chromosome, there was disagreement as to the location of its centromere until differential staining confirmed that the X-chromosome is sub-metacentric (Trujillo et al., 1962; Benirschke et al., 1967; Chandley et al., 1975b; McFeely, 1975; Buckland et al., 1976; Eldridge and Blazak, 1976; Melchior and Höhn, 1976). The Y-chromosome cannot be distinguished in conventionally stained horse chromosome preparations and by custom has been accepted as the 37th acrocentric chromosome (e.g., Basrur et al., 1969; Gluhovschi, 1970). The Y-chromosome is, however, about the sixth smallest acrocentric chromosome (Buckland et al., 1976).

1.7.1.4. Arrangement of the karyotype of the domestic horse

There has been basic agreement over the standard arrangement of E. c. caballus chromosomes. The format generally used has been that of Hsu and Benirschke (1967). For simplicity, and in agreement with the majority of authors, the same karyotype arrangement is used in this Thesis.

When defined by gross morphology there are three groups in the Hsu and Benirschke (1967) arrangement:

Group I: consists of 26 chromosomes in descending order of size by pairs. Pairs 1, 3, 4 and 13 are sub-metacentric and the remaining pairs are metacentric.

Group II: consists of 36 acrocentric chromosomes in descending order of size in 'pairs'.

Group III: consists of the sex-chromosomes.

1.7.2. Abnormal Karyotypes in the Domestic Horse

Karyotypes containing an abnormal number of sex chromosomes have been reported in infertile mares with gonadal dysgenesis (Payne et al., 1968; Chandley et al., 1975b; Hughes et al., 1975a, 1975b; Hughes and Trommershausen-Smith, 1976, 1977; Blue, 1976; Blue et al., 1978; Bruère et al., 1978; Trommershausen-Smith et al., 1979).

Infertility in XX mares with hypoplastic gonads has been reported (Chandley et al., 1975; Hughes et al., 1975b). It was suggested that the infertility in such cases might be associated with an undetected 63,XO line, or as in some humans might be an inherited genetic defect (Trommershausen-Smith et al., 1979).

The XY sex chromosome constitution was reported in non-mosaic infertile mares with gonadal dysgenesis (Chandley et al., 1975b; Hughes and Trommershausen-Smith, 1976; Kieffer et al., 1976; Trommershausen-Smith et al., 1979). Kieffer et al. (1976) considered their case to be testicular feminization, Trommershausen-Smith et al. (1979) suggested their animal was an example of pure gonadal dysgenesis, while Chandley et al. (1975b) proposed that their case might be either. Although of different etiologies both testicular feminization and XY pure gonadal dysgenesis are considered genetic defects (McDonough et al., 1977). In addition, Sharp et al. (1980) reported a fertile H-Y antigen positive mare which they said had a 64,XY

chromosome constitution, although no karyotype was presented in the paper.

Other infertile mares reported with abnormal karyotypes were one Triplo-X (Chandley et al., 1975b) and two mares with mosaic autosomal trisomy (Chandley et al., 1975b; Trommershausen-Smith et al., 1979).

All those chromosomally abnormal mares were found through investigation of their infertility. They presented no other clinical abnormality. One XO/XX mosaic was found in a random survey of yearlings (Walker and Bruère, 1979; Appendix I).

Infertile horses with abnormal sex chromosome constitutions have been identified because of phenotype anomalies (Basrur et al., 1969, 1970; Gluhovschi, 1970; Bouters et al., 1972, 1975; Dunn et al., 1974; Fretz and Hare, 1976; McIlwraith et al., 1976). In this category could be included XX intersexes (Bornstein, 1967; Gerneke and Coubrough, 1970; Bielánski et al., 1977).

The three tables which follow provide summarized extracts of the literature of those reported cases where supporting cytological or morphological information was provided. Table 1-3 consists of XO mares, Table 1-4 non-mosaic/chimaeric horses other than XO mares and Table 1-5 mosaic/chimaeric horses.

It was not possible to assign specific details to the six new gonadal dysgenesis cases of Trommershausen-Smith et al. (1979) because the information provided was not identified with specific animals. Three of these horses were said to be 63,XO/64,XY mosaics and one a 63,XO/64,XX mosaic. Two of the six were investigated because of "short-legged" proportions as foals and four were investigated because of infertility (Trommershausen-Smith et al., 1979).

Unpublished information on XO mares discovered by the Cytogenetics Unit at Massey University is presented in Appendix II.

1.7.3. Infertility and Chromosome Anomalies in the Domestic Horse

1.7.3.1. Infertility: comments on terminology

Defining fertility in a horse as having produced a live foal can be both too broad and too limiting for either an investigation or a discussion of "infertility" in horses. It can be questioned whether animals which never have had the opportunity to prove their fertility can be considered "infertile". There is a

Table 1-3

Summary of published reports of XO mares.

AUTHORS	BREED	AGE (years)	TISSUES	CHROMOSOME COUNTS					KARYO- TYPE	EXTERNAL APPEARANCE	OESTROUS BEHAVIOUR	VAGINA, CERVIX and VULVA	UTERUS	GONADS
				≤ 62	63	64	≥ 65	Total*						
Payne <i>et al.</i> , 1968	TB	12	leucocyte	-	-	-	-	30	63,XO	normal	irregular cycles	normal	normal	normal, follicles, ovulated.
Chandley <i>et al.</i> , 1975b	Welsh pony	2	leucocyte	5	28	-	-	33	63,XO	normal	no cycles	normal	slightly small	very small (< 2 x 1cm) stroma only, no follicles
	TB x ARAB	4	leucocyte	2	47	1	-	50	63,XO	normal	no cycles	normal	small, immature endometrium	Not palpable <u>per rectum</u> .
Hughes <i>et al.</i> , 1975a,b; 1977 and Hughes and Trommershausen- Smith, 1976	ARAB	-	leucocyte	12	29	0	-	41	63,XO	normal 136cm at withers	-	Cervix: os open, flaccid	small, flaccid	small (< 2 x 1cm)
	ARAB	-	leucocyte and skin	6	17	-	-	23	63,XO	normal 133cm at withers	irregular	Cervix: os open, flaccid	small, flaccid, hypo- plastic endometrium	2.6 x 1.3 x 0.8cm undifferentiated stroma
	ARAB	-	leucocyte and skin	7	23	-	-	30	63,XO	normal 137cm at withers	irregular	Cervix: os open, flaccid	small, flaccid, hypo- plastic endometrium	left: 2.8 x 1.2 x 0.7, right: 3.0 x 1.6 x 0.7cm, all stroma
	ARAB	-	leucocyte	5	20	-	-	25	63,XO	small	-	Cervix: os open, flaccid	small, flaccid	small (< 2.0 x 1cm)
	ARAB	-	leucocyte	6	17	-	-	24	63,XO	small	-	Cervix: os open, flaccid	small, flaccid	small (< 2.0 cm)
	ARAB	-	leucocyte	7	26	-	-	33	63,XO	-	-	-	-	Not palpable.
	ARAB	-	leucocyte	6	16	-	-	22	63,XO	135cm at withers	-	Cervix: os open, flaccid	small, flaccid	very small (4 1cm)
	QH	6	leucocyte and skin	5	44	-	-	49	63,XO	140cm at withers	irregular	Cervix: os open, flaccid	small, flaccid	3.0 x 1.5 x 1.5 cm undifferentiated stroma
QH	-	leucocyte	6	11	-	-	17	63,XO	137cm at withers	-	Cervix: os open, flaccid	small, flaccid	small (< 2.0 x 1.5cm)	
Blue <i>et al.</i> , 1978	TB	4	leucocyte muscle fascia mesentery right ovary	13 2 0 0 5	30 26 6 4 15	0 0 0 0 0	0 0 0 0 0	64 28 6 4 21	63,XO	small 146cm at withers	none	Cervix: flaccid	small	3.5 x 2.0 cm
Bruère <i>et al.</i> , 1978	SB	6	leucocyte	7	23	-	-	30	63,XO	normal	none	Cervix: flaccid and open	small	1.0 x 2.0 cm - no follicles palpable.
	TB	7	leucocyte	5	33	-	-	38	63,XO	normal	cycles reported	-	small	1.0 x 1.0 cm - no follicles palpable.
	TB	6	leucocyte	4	16	-	-	24	63,XO	normal 156.9cm at withers	none reported	Cervix: flaccid and open	small, flaccid	very small - no follicles palpable.
	TB	6	leucocyte	9	20	-	-	29	63,XO	normal	-	Cervix: flaccid	small	very small
	TB	9	leucocyte	3	47	-	-	52	63,XO	small thick crest	indefinite	Cervix: open	very small	very small

Key: TB = Thoroughbred, QH = Quarter horse, SB = Standard Breed

*Total = total cells evaluated

Table 1-4
Summary of published reports of horses with mon-mosaic/chimaeric sex chromosome abnormalities, other than XO mares.

AUTHORS	BREED	AGE (years)	TISSUES	CHROMOSOME COUNTS					Total*	KARYO-TYPE	EXTERNAL APPEARANCE	OESTRUS and BEHAVIOUR	VAGINA, CERVIX and VULVA	UTERUS	GONADS
				52	53	64	65	66							
Chandley <i>et al.</i> , 1975b	TB	8	leucocyte	0	1	37	0	0	38	64,XY	normal female	no cycles	normal	small	very small (<2 x 1cm)
Hughes & Trommershausen-Smith, 1976	pony	-	leucocyte	0	15	31	0	0	46	64,XY	normal female	-	Cervix: os open, flaccid	small and flaccid	very small (< 1cm)
Kieffer <i>et al.</i> , 1976	QH	7	leucocyte testis	0	0	120	0	0	120	64,XY	female	no oestrus/ stallion-like	vagina present no cervix	none	testis in abdomen 6 x 3 x 3 cm
Trommershausen-Smith <i>et al.</i> , 1979	ARAB	5	leucocyte	-	-	-	-	-	-	64,XY	female	-	-	-	-
	SHETLAND	>6	leucocyte	-	-	-	-	-	-	64,XY	female	cycling	Cervix flaccid	small prepubertal	very small (10 x 5 x 3 mm)
Sharp <i>et al.</i> , 1980	-	5	leucocyte skin	-	-	100	-	-	100	64,XY	female	cycling (dam of filly)	-	underdeveloped	small (< 2 x 2 cm)
Bornstein, 1967	ARDANT	1	skin	3	1	12	1	2	19	64,XX	ambiguous male enlarged clitoris	-	vulva present	present	hypoplastic testicles
	N. SWED.	1½	skin mullerian duct	5	2	38	2	0	47	64,XX	ambiguous male penis-like organ	stallion behaviour	vulva present	present	intra-abdominal hypoplastic testicles
Gerneke & Coubrough, 1970	ARAB	1	bone marrow	-	-	-	-	-	-	64,XX	ambiguous male penis-like clitoris	-	rudimentary vulva	-	hypoplastic inguinal testicles
Bielanski <i>et al.</i> , 1977	-	>4	leucocyte gonad	-	-	60	-	-	60	64,XX	ambiguous female small penis	stallion behaviour	not present	not present	small gonad in abdomen
Gluhovschi <i>et al.</i> , 1970	NONIUS	3	leucocyte	-	-	-	-	63	63	66,XXX	ambiguous male glans penis	slightly aggressive	-	infantile	testis: inguinal and hypoplastic
Chandley <i>et al.</i> , 1975b	TB	5	leucocyte	-	-	-	28	-	30	65,XXX	normal female	irregular oestrus	normal	small, immature, with hypoplastic endometrium	small (< 2 cm), one follicle once, biopsy showed only stroma.

Key: TB = Thoroughbred, QH = Quarter Horse, N. Swed. = North Swedish Breed
* Total = Total cells evaluated.

Table 1-5

Summary of published reports of horses with mosaic/chimaerism.

AUTHORS	BREED	AGE (years)	TISSUES	CHROMOSOME COUNTS					KARYOTYPE	EXTERNAL APPEARANCE	SEXUAL BEHAVIOUR	INTERNAL GENITALIA	GONADS	
				62	63	64XX	64X	65						Total*
Chandley <u>et al.</u> , 1975b	ARAB	5	leucocyte	3	35	12	-	-	50	63,XO/64,XX	normal	no cycles	normal	very small (2 x 1cm)
	TB	7	leucocyte	7	22	3	-	1	33	63,XO/64,XX	slightly gelding-like	mild after irrigation	Cervix pale and slightly flaccid; Uterus, small.	very small (2 x 1 cm) no follicles
Hughes <u>et al.</u> , 1975, 1977	APPL.	6	leucocytes and skin	-	13	25	-	-	38	64,XX/63,XO	normal 135 cm at withers	irregular oestrus	Cervix firm, os closed; Uterus flaccid, endometrium hypoplastic	very small (1.8 x 1.3 cm) no follicles
Bruère <u>et al.</u> , 1978	TB	9	leucocyte	5	21	36	-	-	62	64,XX/63,XO	normal	no oestrus 'resented teaser'	normal	small, follicular structure on one ovary
Walker & Bruère, 1979	TB	1	leucocyte	3	29	12	-	-	44	63,XO/64,XX	normal	-	-	-
Hughes <u>et al.</u> , 1977	QH	-	leucocyte	-	8	-	24	-	32	64,XY/63,XO	-	-	Cervix flaccid, os open; Uterus small and flaccid	small (2.0 x 1.5 cm)
McIlwraith <u>et al.</u> , 1976	½ ARAB	3	gonads skin	-	-	61	26	-	185	64,XX/64,XY	cryptorchid with penis	stallion-like with erections	-	ovarian and testicular
Bouters & Vandeplasche <u>et al.</u> , 1972; Bouters <u>et al.</u> , 1975	Welsh pony	2	-	-	-	190	-	10	200	64,XX/65,XXY	vulva and penis	stallion-like	-	hypoplastic abdominal testis
Dunn <u>et al.</u> , 1974	BELGIAN	2	leucocyte skin gonad	13 8 31	12 5 12	18 15 17	1 -	3 1 4	55 30 76	64,XX/65,XXY/ 64,XY	cryptorchid	stallion-like	not detected	testicular with no germ cells
Basur <u>et al.</u> , 1969, 1970	CLYD. x PERCH.	4	gonad (sex chromatin marrow/blood)	36	8	32	19	3	108	64,XX/64,XY/ 65,XXY/63,XO	male cryptorchid vulva displaced	stallion-like	none	agonadal processus
Fretz & Hare, 1976	ARAB	½	leucocyte, gonad and epididymis	17	38	158	-	13	241	64,XX/65,XXY/ ?63,XO	female with enlarged clitoris	stallion-like	no uterus	testicular with no germ cells
Chandley <u>et al.</u> , 1975b	TB	15	leucocyte	1	7	45	-	2	55	64,XX/65,XX+ autosomal fragment	normal	regular oestrus	normal Uterus small	very small (2 x 1cm) no oocytes
Trommershausen-Smith <u>et al.</u> , 1979	ARAB	2	-	-	-	-	-	-	-	64,XX(del 2q-)	small	irregular oestrus	-	ovaries active twice, age 4 and 5 years.

Key: TB = Thoroughbred, QH = Quarter horse, Appl. = Appaloosa, Clyd. = Clydesdale, Perch. = Percheron

*Total = Total cells evaluated

group of horses known to the breeding industry as "shy breeders". Traditionally these horses have difficulty in generating foals. However, some of these "shy breeders" are fertile and will breed successfully under range conditions, or when artificial insemination procedures are used, but neither of these systems is acceptable in the management of registered Thoroughbreds.

Human Geneticists sub-divide infertility into: sterility, the inability to form viable gametes; infertility, the assumed or known loss of products of conception; and sub-fertility, the failure or inability to efficiently reproduce as measured by live offspring or as determined by a physical or anatomical defect such as a non patent Fallopian tube (Opitz et al., 1979). An absolute division into these categories, even in humans, is usually not possible in individual cases. In the female, sterility can seldom be differentiated from infertility. Sub-fertility, on the other hand, implies some standard of fertility that may be purely subjective or qualitative.

Whenever possible, however, an attempt has been made in this Thesis to follow human genetic terminology with reference to "infertility", "sterility", and "sub-fertility".

1.7.3.2. Chromosome anomalies in the domestic horse

It was obvious that the cases reported in the literature could not reflect the true incidence of chromosome abnormalities in the horse population. This was because there are so few facilities available where cytogenetic analysis of horses can be attempted (Blue et al., 1978; Bruère et al., 1978; Walker and Bruère, 1979).

The majority of the animals reported in the literature were presented for cytogenetic analysis because of infertility, either with or without associated phenotype abnormalities. As a rule, animals with phenotype abnormalities are culled from horse populations by breeders, while infertility is a notoriously biasing factor in identification of chromosome abnormalities (e.g., Dewhurst, 1978). The notable absence of phenotypically normal stallions in the reports emphasizes both these points, but particularly the latter. The majority of male horses are gelded so their fertility or potential fertility is never questioned.

However, support for the theory that there are large numbers of chromosomally abnormal animals in the equine population can also

be found within the literature. Besides the fertile XY mare (Sharp et al., 1980), none of the infertile mares reported on Tables 1-4 and 1-5 were said to have had some signs of oestrus or follicular activity (Payne et al., 1968; Hughes et al., 1975a, b; Chandley et al., 1975b; Hughes and Trommershausen-Smith, 1976; Bruère et al., 1978; Trommershausen-Smith et al., 1979). Therefore, some of these animals may belong to the group of "shy breeders", which with therapy may have been fertile.

CHAPTER II

MATERIALS AND METHODS

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CHAPTER II

MATERIALS AND METHODS

2.1. Subjects

The horses discussed in this Thesis are described in detail at the beginning of their Chapter or section.

These horses were investigated for cytogenetic abnormalities for different reasons. H-11, a stallion, was referred by his veterinarian because of suspected abnormal rate of growth (Chapter III). H-46, a racing mare, was investigated at the request of her trainer who was convinced she showed "male traits" (Chapter V, section 5.2.2.) H-27, a mare with chronic endometritis, was part of a routine sampling of animals admitted to the Large Animal Unit at Massey University (Chapter VI). H-52, an infertile mare, was referred to the Cytogenetics Unit, Department of Veterinary Clinical Sciences, Massey University, by her practitioner as a suspected XO mare. H-47, a two year old maiden mare, was investigated at the author's request because of her congenital defects (Chapter V, section 5.2.3.).

2.2. Cytogenetic Techniques

The details of the procedures used can be found in Appendix VI and those of the materials and supplies used in Appendix V. Terms used throughout this Thesis are those proposed by the Tissue Culture Association (Schaeffer, 1979).

2.2.1. Leucocyte Cultures

Samples of heparinized whole blood were cultured according to standard micro-culture procedures as described in Appendix VI.

2.2.2. Cell Cultures Other Than Leucocyte Cultures

Either standard trypsin digestion or standard plasma clot techniques were employed to establish primary cultures. Description of the tissue(s) used from primary cultures, the technique(s) used in establishing them and any deviations from the detailed procedures listed in Appendix VI are provided in the materials and methods section of each Chapter.

Cytogenetic analysis of cells from explant cultures was usually performed on lines with the lowest passage number, progressing upward in passage number until sufficient cells had been evaluated. When a higher numbered passage had a shorter total time in culture than a lowered numbered one, cells from the culture with the shortest total culture time were analysed. Trypsin digestion cultures were harvested as primary cultures. Except as noted in the text, analysis was performed on cells from cultures with less than four passages and on cells from cultures whose total elapsed culture time was less than four weeks.

2.2.3. Harvesting Cell Cultures and Slide Preparation

Standard procedures for harvesting mitotic cells from cell cultures were used. Slides were air-dried.

2.2.4. Staining

Conventional Giemsa stain was used throughout this study for chromosome counts and karyotyping. Differential staining techniques were applied for confirmation of H-68 (Chapter III) and H-52 (Chapter IV). Details of both C-band and G-band techniques can be found in Appendix VI. For G-banding initially a proteolytic method was used; later an acetic-saline method was found to yield better results.

2.2.5. Sex-Chromatin

Samples of tissue from the cerebellum and spinal cord were investigated for sex-chromatin from H-52 (Chapter IV) and H-27

(Chapter VI). The tissue was prepared according to the method of Bruère (1966), by the Histology Preparation Unit, Faculty of Veterinary Science, Massey University.

Cells were evaluated only if they showed a well defined nucleus and nucleolus. Details of the evaluation of sex-chromatin and the preparation of material can be found in Appendix VI.

2.2.6. Evaluation of Cultured Cells

Several procedures have been suggested to prevent misinterpretations of in-vitro cultured cell results (e.g., Polani, 1961; Reitalu, 1967; Littlefield and Mailhers, 1975; Hassold et al., 1978; Wahlström, 1978). Among these were the use of "in-situ" techniques with comparison of growth centers, making parallel cultures from the same specimen and analysing each separately, sampling various tissues and areas of those tissues, making repeat samples whenever possible and performing analysis on cells from cultures with the shortest possible culture time.

Unfortunately, the "in-situ" technique was found to be impractical in the horse, the cultured cells of which are unsuitable for sex-chromatin study and the high diploid chromosome number prevented "in-situ" karyotype evaluation. However, all the other procedures were applied to cultured cells during this study.

Specific problems of seeming aneuploidy and polyploidy encountered during this investigation are discussed in the relevant Chapters and in the General Discussion. As a general rule a culture specific rate of artifactual chromosome loss was estimated for each culture and aneuploid cells in excess of that amount were considered to indicate the presence of such a cell line. The criteria for acceptance of a metaphase with extra chromosomes was metaphase and chromosome morphology and secondly the identification of all the chromosomes.

Metaphases were selected for evaluation on the basis of metaphase and chromosome morphology. Identification of all non-acrocentric chromosomes had to be possible, that is a "differential count". Evaluation was by differential counts and in selected instances, by karyotype analysis.

CHAPTER III

H-11, A FERTILE XY/XYY/?XO MOSAIC/CHIMAERIC STALLION

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CHAPTER III

H-11, A FERTILE XY/XYY/?XO MOSAIC/CHIMAERIC STALLION3.1. Introduction

The XYY chromosome configuration is thought to occur in one to three of one-thousand human births (Boué and Boué, 1974) and has been infrequently reported in mice (Evans et al., 1978). Neither the XYY condition nor the presence of a mosaic/chimaeric XYY cell line had been reported in E. c. caballus.

H-11, a Thoroughbred colt, was referred for cytogenetic analysis at the age of nine months because his rate of growth seemed accelerated. Since abnormal growth rate is associated with both XXY and XYY boys (Leonard et al., 1974; Ratcliff, 1976; Hudson and Robinson, 1977),

H-11's veterinarian was suspicious that H-11's rate of growth might be symptomatic of a sex-chromosome abnormality.

On the basis of the initial lymphocyte culture, H-11 was diagnosed as a 64,XY stallion with probable low level 65,XYY mosaic/chimaerism. Despite the extreme cooperation of both his owner and his veterinarian, any cytogenetic follow-up of H-11 was difficult. He was over 300 miles from the laboratory and at an inconvenient distance from the nearest airport for collection and dispatch of samples.

Intensive cytogenetic studies of H-11's relatives and progeny were also hindered by transportation difficulties. In addition, there was an understandable reluctance by some owners and breeders to have

their animals associated in any way with an investigation, the results of which might be economically damaging. When it was possible to perform cytogenetic studies of relatives or progeny of H-11, it was necessary to limit the analysis to screening for non-mosaic/chimaeric karyotype abnormalities.

The primary importance of H-11 was, and is, considered to be some possible manifestations and implications of his mosaic/chimaeric condition, rather than the nature of the condition in him. He was the youngest animal diagnosed with a chromosome abnormality. His development could be observed and he was also potentially fertile and his reproductive performance could be monitored. Moreover, since he was under observation and was subject to controlled breeding, he might be of value in equine genetic studies¹.

¹H-11 is currently involved in a private breeding project concerned with breed improvement and the inheritance of coat colour. Both his present and former owner (who maintains stud rights) have discussed proposed genetic programmes with the author, and both have stated their interest in continued cytogenetic monitoring of H-11's progeny whenever possible.



Figure III.1 H-11 as a four year old.

3.2. Materials and Methods

3.2.1. Sources of Information

The primary sources of information on H-11 and his progeny were the records of his veterinarian and the records at his first stud. Information on H-11's registered Thoroughbred relatives was obtained from official breed publications. These sources were supplemented by observations and interviews conducted in person during three brief visits to H-11's area, and by telephone conversations with persons² in several regions of New Zealand who either were or had been directly involved with the various horses. Anecdotal information was reported only when it was provided by at least two persons independently. Whenever possible such information has been verified through official records.

3.2.2. H-11

3.2.2.1. Description

Sex:	male
Foaled:	1975
Breed:	Thoroughbred
Colour:	chestnut
Size:	176.25 cm (17.2½ h.h.) at withers (in 1978)
Age of sire at birth:	8 years
Age of dam at birth:	unavailable

H-11 is shown in Figures III.1 and III.3a.

3.2.2.2. General history

At parturition, following a normal gestation, the dam of H-11 ruptured along the perineum and her intestines prolapsed. The mare was destroyed and the foal delivered by salvage Caesarian.

The foal was large at birth. His testicles were considered to be larger than usual by those present at his delivery, but they

²veterinarians, owners, estate managers and persons providing services to the breeding industry who wished to remain anonymous.

were not measured. As a foal H-11 had no medical problems except dermatophilosis and ryegrass staggers, which were common in the foals in his region.

At nine months of age H-11 measured 163.75 cm at the withers (16.1½ h.h.) and at three years 175 cm (17.2 h.h.). He stood 176.25 cm (17.2½ h.h.) in August of 1978. X-rays taken in April, 1979, confirmed that the radial epiphyseal plates had closed.

H-11 had a placid temperament. According to his owner he showed better than average intelligence in "schooling" and it was independently supported that he was a promising show jumper. H-11 displayed normal male behaviour and never lacked libido.

3.2.2.3. Reproductive performance

The majority of H-11's progeny were sired under contractual agreements which stipulated what information could be disseminated by the stallion's owner. During 1979 H-11 was sold and access to information on mares and progeny was difficult to obtain. Even when a mare's owner agreed to divulge information, it was with the condition that the mare's identity be masked. As a result, letters of the alphabet have been used to designate individual mares and in all but a few cases no identifying information (i.e., age, breed) is provided.

In 1977 H-11 served four mares. Mare "D" was 16 years old and although diagnosed as pregnant, when she subsequently died she was discovered to not be in foal and to have had advanced ovarian neoplasm. The other three mares delivered live foals, which were examined for simple chromosome anomalies (section 3.4.).

H-11 served six mares in 1978. Mares "B", "E" and "F" were diagnosed pregnant by palpation per rectum on the 42nd day after a single service and subsequently delivered live foals. Mares "G" and "K" were served once, but were sold before reliable manual pregnancy testing could be done at the stud. However, both these mares were reported to be in foal by veterinarians who examined them between 30 and 40 days following service. Mare "G" was still in foal at 10 months. Mare "K" produced an XO filly (H-68). Mare "J" could not be manually tested for pregnancy and a blood test was negative. However, it was

reported that "J"'s owner claimed that both "J" and a pregnant mare with whom "J" shared a paddock aborted on the same day.³

Prior to taking up stud duties on a new property, H-11 served seven mares. Mares "B", "N" and "P" were synchronized with prostaglandins prior to service. Mare "P" who was too wild to manually pregnancy test and mares "Q" and "R" who were too close to the time of service for reliable pregnancy diagnosis per rectum were entered in the stud records as pregnant by reason of non-return to oestrus. Pregnancy was accepted by their owners on that basis. H-11 only served each of these mares once, so they were considered to have conceived to first service.

Table 3-1 summarizes the reproductive performance of H-11 and includes information on the progeny.

3.2.2.4. H-11's sire and dam

The dam of H-11 was an unregistered Thoroughbred mare. She was known to have had a live colt foal, which as a mature gelding stood 172.5 cm (17.1 h.h.) at the withers. Unfortunately, little information was available on this mare, but she was 'well bred' on her sire side.

H-11's sire was a registered Thoroughbred stallion with a good breeding record. According to the Register of Thoroughbred Stallions of New Zealand, he had an average estimated conception rate of 89% and average live foal rate of 67% (over 43 mares). He was related, within three generations, to five confirmed XO mares including his grandfilly (H-68). However, this was probably because he, like most mares that had been examined for cytogenetic abnormalities, had a very good pedigree.

3.2.3. Relatives and Progeny Investigated

The history of H-11's sire is given in section 3.2.2.4. No information was available on the two half-brothers from which blood was obtained, except that they had been gelded.

³There was a belief in this area in a specific environmental cause of abortions. It was not possible to substantiate suggestions made in this regard for this property.

Table 3-1

Reproductive performance of H-11 (1977-1980).

YEAR of Service	MARE	BREED of mare	PROGENY	COMMENTS
1977	"A"	SB	colt	H-57: Premature foal, with persistant <u>Ductus arteriosus</u> .
1977	"B"	pony	filly	H-58: Large for age and size of dam at about 6 months. Figure III.3b taken at age 9 months.
1977	"C"	TB	filly	H-59: Normal for age.
1977	"D"	-	-	Mare died. She had advanced ovarian cancer.
1978	"B"	pony	filly	Persistant <u>Ductus arteriosus</u> . (Figure III.2 at age 3 days)
1978	"E"	-	colt	Foal said to be large for age.
1978	"F"	-	colt	Foal said to be large for age.
1978	"G"	-	-	Mare was still pregnant at 10 months.
1978	"J"	-	-	Mare may have aborted, if so possibly due to environmental factor.
1978	"K"	TB	filly	H-68: Stillborn XO mare.
1979	"M"	-	-	Positive pregnancy test
1979	"J"	-	-	Positive pregnancy test
1979	"B"	pony	-	Positive pregnancy test
1979	"N"	-	-	Positive pregnancy test
1979	"P"	-	-	Non-return to oestrus
1979	"Q"	-	-	Non-return to oestrus
1979	"R"	-	-	Non-return to oestrus

SB = standard breed, TB = Thoroughbred



Figure III.2 Mare "B" with three day old 1979 filly by H-11. Mare "B" was the dam of H-58, shown in Figure III.3b.

H-57 was a colt out of a Standard Breed mare. He was foaled prematurely. At approximately two months of age, when he was bled for cytogenetic analysis, he was considered to be small for his age. He had suffered a persistent Ductus arteriosus which had closed when he was ten days old. H-57 was destroyed before he was 12 months old because of a compression fracture of the hock. He had the tall socks of his sire and a face blaze.

H-58 was a filly out of a pony mare. Her dam, mare "B" in section 3.2.2.3., is shown in Figure III.2 with H-58's full-sister. H-58 is shown in Figure III.3b. She was a few centimeters shorter than her dam by the age of 6.5 months and was taller than her dam by the age of nine months when the photograph (Figure III.3b) was taken.

H-59 was a filly out of a Thoroughbred mare. She was of normal development for her age. H-59 had tall socks like her sire and a face blaze.

H-68, an XO filly, was stillborn on 12th October, 1979. She was a full term conceptus, delivered in breech presentation. "Blood filled the abdominal cavity and the liver capsule had ruptured. Death was probably due to internal haemorrhage caused by the dystocia" (H. Dewes, pers. comm.). The dam of H-68 was 13 years old at the time of foaling. A large, unregistered Thoroughbred, the dam had had at least four live foals, by four different sires, previous to H-68's still-birth. According to stud records, the dam's last delivery before that of H-68 occurred without assistance, from the standing position at midday in an open paddock. H-68 was conceived at the "foal heat" following that delivery.

3.2.4. Cytogenetic Materials and Methods

Heparinized blood samples were refrigerated or were packed in ice as soon as possible (no more than four hours) after collection. They were then dispatched by the earliest scheduled air service, which was not less than 12 hours or more than 24 hours after sample collection. The skin sample was excised from H-11's foreshoulder, placed in sterile saline supplemented with antibiotics, and was refrigerated overnight before it was carried by air to Palmerston North.

Multiple plasma clot cell cultures were initiated from that sample. In the absence of homologous plasma, serum from heparinized

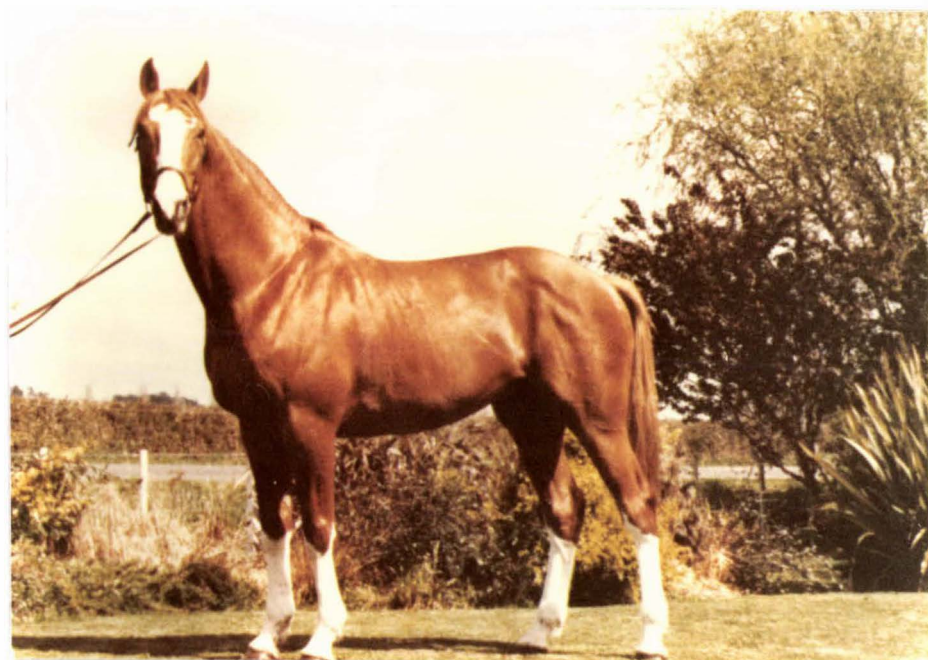


Figure III.3a H-11, the sire of H-58.

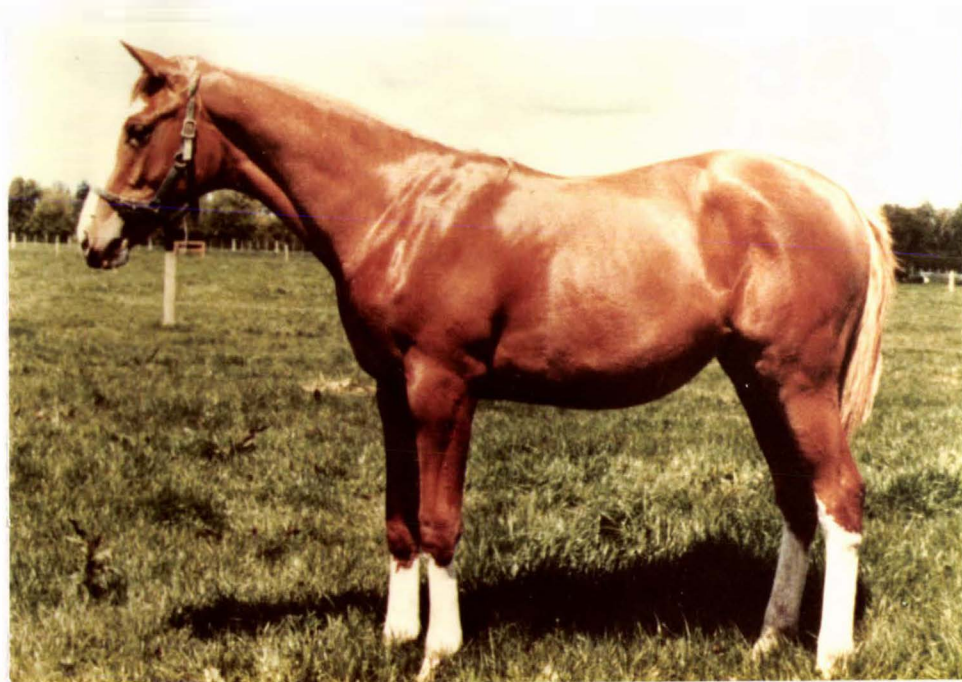


Figure III.3b H-58 age nine months. She measured 156.25 cm at withers ($15.2\frac{1}{2}$ h.h.). Note the strong resemblance to her sire.

whole blood (the residue was used as Blood III culture) and in three flasks sterile sheep plasma was used for the plasma clots. There was no apparent difference between the two culture sets in either viability or growth.

An attempt was made to dissect 'epidermis' from 'dermis' and these separate cell cultures were initiated and maintained. Both cultures appeared to have only fibroblast-like cellular morphology, however, 'dermis' cultures were the first to show out-growth from the explants and persisted longer in culture. The chromosome preparations from these two 'tissues' were analysed separately, but no difference between them was found in the proportions of cell types present, so the results were combined. The chromosome preparations analysed were from passages one through three, which had been in culture less than four weeks.

Cell cultures of the 6th passage were analysed from H-68. This culture had been initiated from a piece of kidney by G. Horner and R. Hunter. A fourth passage monolayer culture was flown to Palmerston North in an insulated container maintained within physiological temperature range.

Except as noted above, all cell culture procedures were as described in Appendix VI.

3.3. Results of Cytogenetic Analysis of H-11

Despite difficulties in transporting samples to the laboratory for processing, it was possible to obtain three blood samples and a skin sample from H-11. A total of 268 metaphases, 167 from leucocytes and 101 from skin, were evaluated from cell cultures initiated from these samples.

Results from the first lymphocyte culture (Blood I) were considered conclusive that H-11 was not a simple (non-mosaic/chimaeric) XXY or XYY animal, and were strongly indicative that he was a mosaic/chimaera for a cell line with 65 chromosomes, 38 of which were acrocentric. By convention, in the absence of differential staining, the 38th acrocentric was accepted as a supernumerary Y-chromosome, and the line was designated "XYY". Confirmation of 64,XY/65,XYY mosaic/chimaerism was based on the second leucocyte culture and subsequently cytogenetic evaluation of cultured skin cells. Because H-11 was a valuable animal, tissues available for investigation were limited and the extent of his mosaic/chimaerism could not be estimated.

Table 3-2 summarizes the results of cytogenetic investigations of cells from H-11. Examples of a normal 64,XY cell (Figure III.4), two cells with 65 chromosomes (Figures III.5 and III.6), and a cell with 63 chromosomes (Figure III.7) follow Table 3-2.

Low level XY/XYY mosaic/chimaerism was estimated to have been present in H-11's leucocytes and skin. Of the cells evaluated that had counts of either 64 or 65 chromosomes, approximately 8-10% had 65 chromosomes. The actual percentages of the cells analysed from H-11 that contained 65 chromosomes were; overall 7.46%, leucocytes 7.8% and cultured skin 6.9%. These percentages from H-11's cultured cells were respectively 1.73, 2.0 and 1.4 times those found by Blue (1976) in cultured cells from 'normal' stallions (overall 4.3%, leucocytes 3.9% and skin 4.8%). But the percentages of cells with 65 chromosomes found in H-11 did not exceed those reported by Blue (1976) for some of the individual 'normal' stallions (20-33% of cells counted per animal) he studied. Some of those 'normal' stallions may have been mosaic/chimaeras. Therefore, the actual number of cells with 65 chromosomes found in H-11 was probably nearly totally reflective of mosaic/chimaerism for a cell line containing 65 chromosomes, as ascertained through tissue culture techniques.

Table 3-2a

The distributions of chromosome counts
from H-11's cultured cells.

TISSUES	DISTRIBUTION OF GROSS COUNTS						TOTAL CELLS
	≤ 62	63	64	65	>65	Polyploid	
Blood I (1977)	5	16	60	6	-	6	93
Blood II (1978)	1	5	43	5	-	1	55
Blood III (1979)	4	4	9	2	-	-	19
Total Lymphocytes	10	25	112	13	-	7	167
Skin	10	10	66	7	1	7	101
Total	20	35	178	20	1	14	268
(%)	(7.5)	(13.1)	(66.4)	(7.46)	(0.4)	(5.2)	

Table 3-2b

The distributions of differential chromosome
counts from H-11's cultured cells.

TISSUES	DISTRIBUTION OF DIFFERENTIAL COUNTS						TOTAL CELLS	
	≤ 62	63	64	65	>65	Polyploid		
	(-)	(26)	(27)	(27)	(27)	(**)		
Total Lymphocytes	10	9	16	112	13	-	7	167
Skin	10	3	7	66	7	1	7	101
Total	20	12	23	178	20	1	14	268
(%)	(7.5)	(4.5)	(8.6)	(66.4)	(7.46)	(0.4)	(5.2)	

** One cell 86(29)

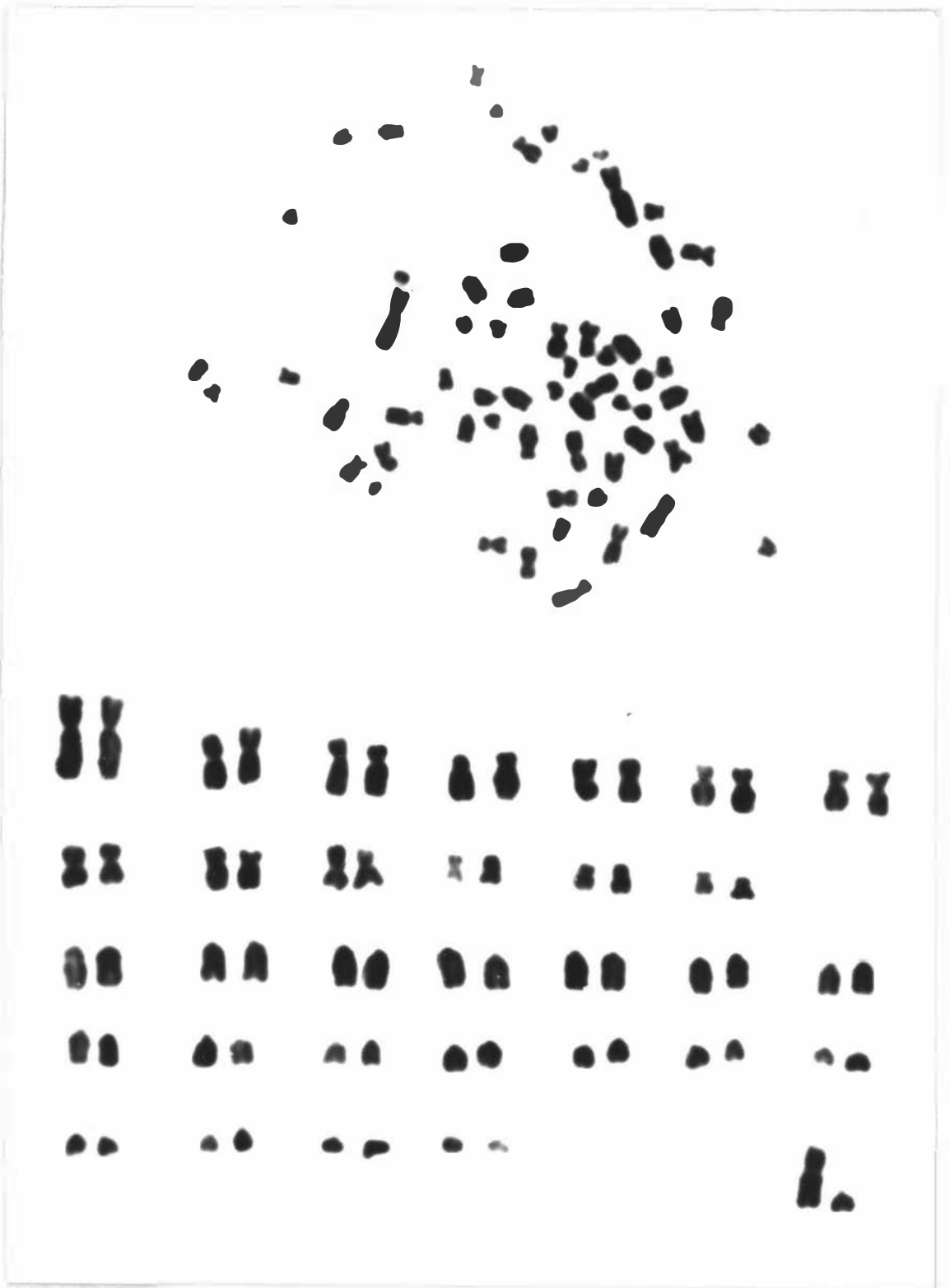


Figure III.4 A normal 64,XY metaphase from H-11 (approx. X 5,000).

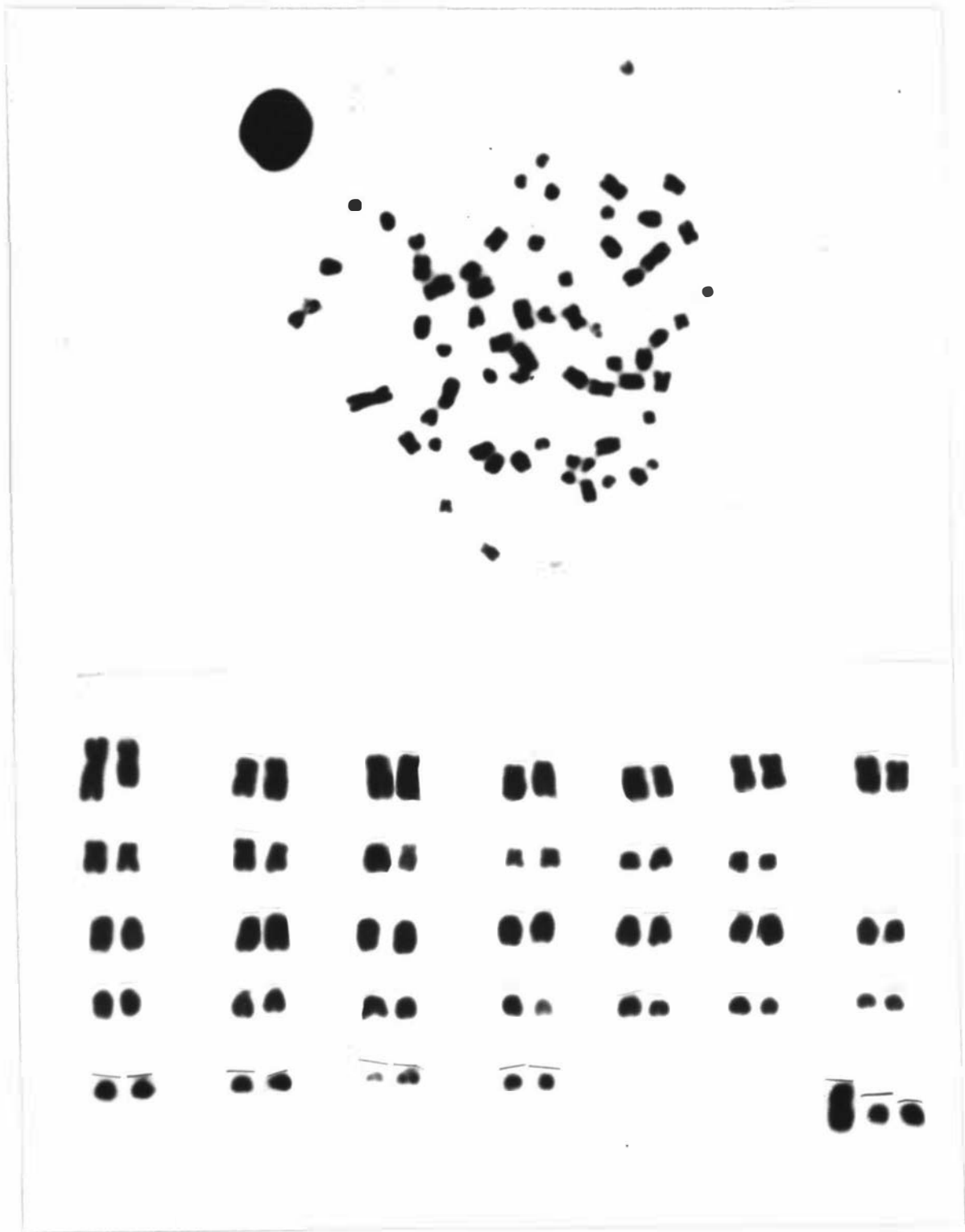


Figure III.5 A metaphase with 65 chromosomes from H-11's cultured skin. By convention the 37th and 38th acrocentric chromosomes were designated "Y" chromosomes. (Approx. X 5,000)

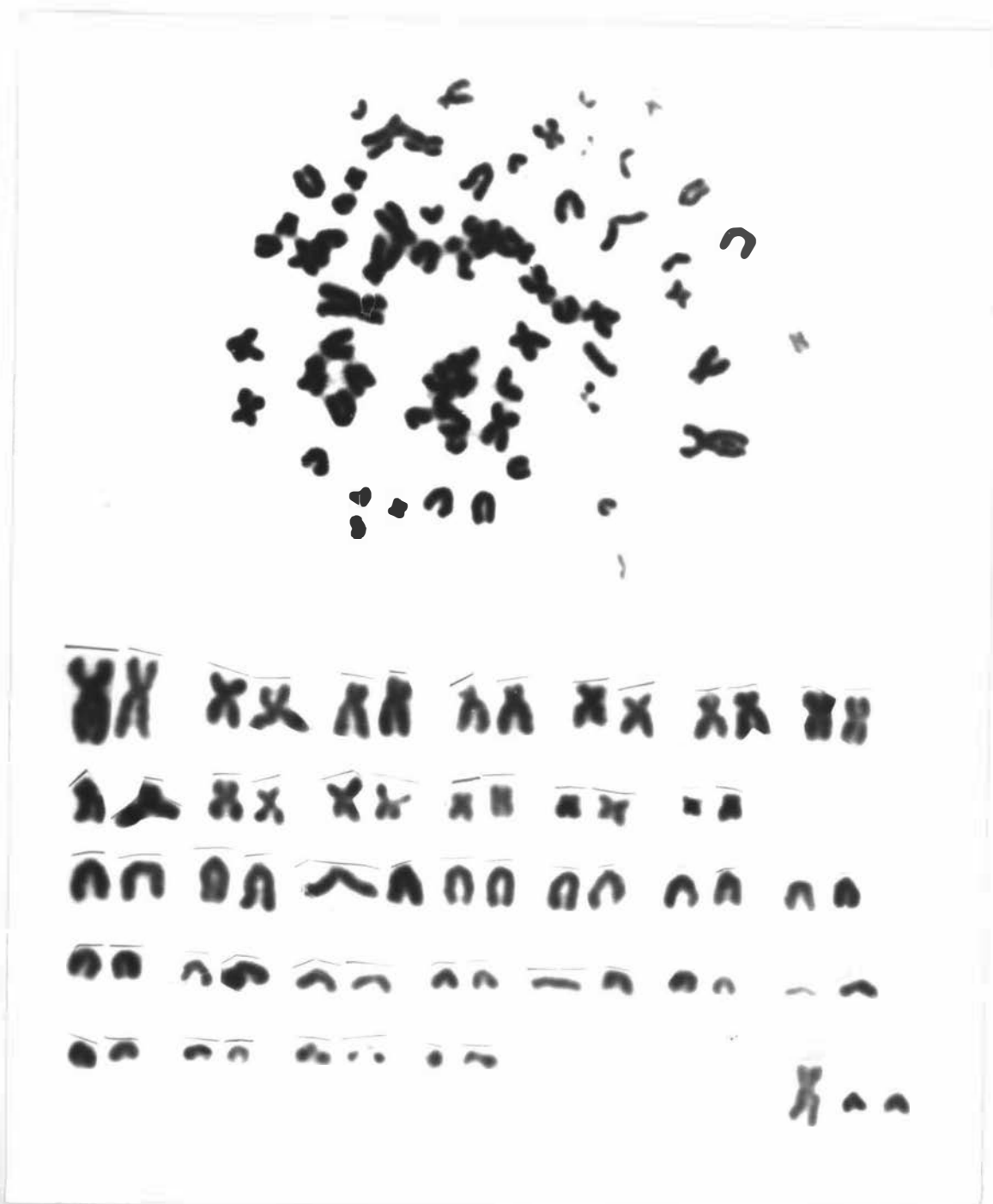


Figure III.6

A metaphase with 65 chromosomes from H-11's cultured lymphocytes. By convention the 37th and 38th acrocentric chromosomes were designated "Y" chromosomes. (Approx. X 5,000)

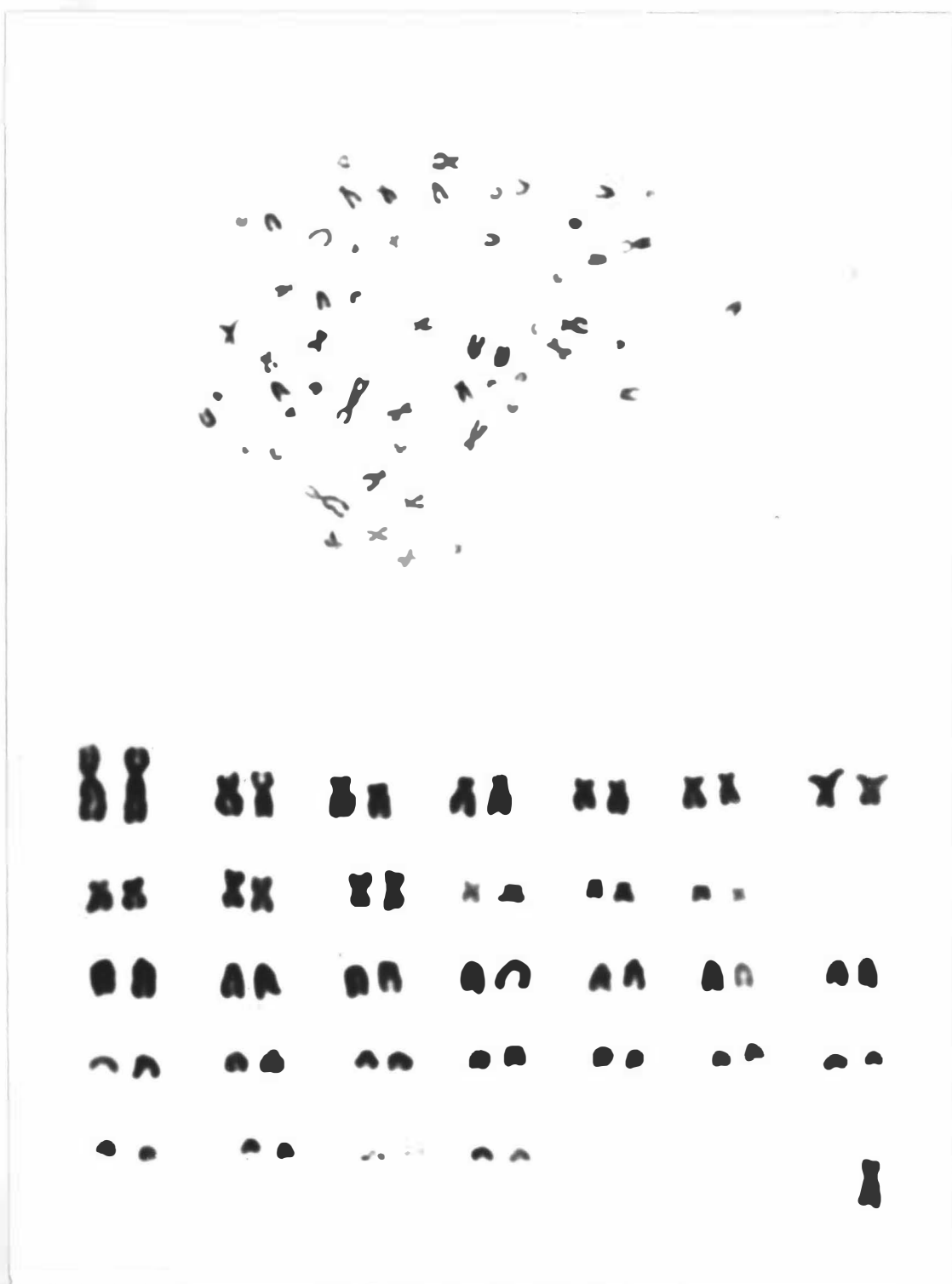


Figure III.7 An example of a 63,XO cell from H-11 (approx. X 5,000).

Without banding, the metaphases with 63 chromosomes, 27 of which were non-acrocentric, could not be identified as XO cells rather than cells which had lost through artifact any of the 37 acrocentric chromosomes. Only in Blood II (Table 3-2) did the number of cells with possible 63,XO configuration exceed an estimated culture specific rate of artifactual chromosome loss, e.g., percentage of cells counted with ≤ 62 plus 63/26 non-acrocentric chromosomes. Combining cells with chromosome counts of 63 from H-11, that is probable artifactual 63/26 non-acrocentric metaphases and metaphases with counts of 63/27 non-acrocentric chromosomes which included true XO cells, 13.1% of the cells evaluated from H-11 had 63 chromosomes. Overall, 9.9% of the cells evaluated by Blue (1976) from 'normal' stallions had 63 chromosomes.

In both H-11 and Blue's (1976) 'normal' stallions, the percentage of cells evaluated with less than or equal to 63 chromosomes were approximately 20% ($\pm 0.5\%$). Although the percentage of cells with 63 chromosomes was not appreciably higher in H-11 than that found by Blue (1976), he did not report differential (acrocentric/non-acrocentric) counts, and un-investigated⁴ mosaic/chimaerism may also have elevated his rate of cells with 63 chromosomes in 'normal' stallions.

It was thought that the slightly higher rate of cells with 63 chromosomes in H-11 compared to that found by Blue (1976) and the excess of cells with 63/27 non-acrocentric chromosomes in Blood II may have reflected a minor mosaic/chimaeric XO line in H-11. However, while it is thought probable that H-11 carried a 63, XO line, designation of him as a 64,XY/65,XYX/63,XO mixoploid was considered to be unconfirmed.

3.4. Extended Cytogenetic Study of H-11: His Relatives and Progeny

A single sample of blood from H-11's sire (H-39) and two of his half brothers (H-40 and H-41) became available for culture and chromosome evaluation. In addition, H-11's three 1978 foals (H-57, H-58 and H-59) were examined for non-mosaic/chimaeric sex chromosome anomalies. Cells from the kidney from H-68, H-11's 1979 stillborn XO filly, were also evaluated.

⁴ Blue (1976) was investigating simple (non-mosaic) chromosome abnormalities in the horse. Therefore, 'normal' was defined as having a modal chromosome number of 64.

A total of 243 metaphases were analysed from cultured cells from H-11's relatives and progeny. The results of these cytogenetic investigations are given in Table 3-3.

Table 3-3

Results of cytogenetic investigations of H-11's relatives and progeny.

HORSE	KARYOTYPE	DISTRIBUTION OF DIFFERENTIAL COUNTS							TOTAL CELLS	
		≤62	63		64		65	Other		P*
		(-)	(26)	(27)	(27)	(28)	(**)	(***)		(-)
Sire										
H-39	64,XY	5	3	2	49	-	2	-	-	61
½ Brothers										
H-40	64,XY	5	-	3	33	-	-	-	1	42
H-41	64,XY	4	4	1	31	-	-	2	-	42
Progeny										
H-57	64,XY	3	1	1	12	-	-	-	1	18
H-58	64,XX	1	-	-	-	14	-	-	-	15
H-59	64,XX	1	-	-	-	12	-	2	-	15
H-68	63,XO	9	-	36	-	-	-	-	5	50

* P = polyploids. Cells were recorded only when they could be karyotyped.
 ** See text, presumptive 65,XYX cells.
 *** See text, ambiguous cells suggestive of possible cytogenetic irregularity.

Sixty-one cells were evaluated from one leucocyte culture from H-39 (H-11's sire). Two cells were found with chromosome counts of 65/38 acrocentrics, including the one presented in Figure III.8 which contains a tri-radial chromosome arrangement. Therefore both the possibility of mosaic/chimaerism for a line containing 65 chromosomes and the possibility of a mitotic chromosome instability were suggested in H-11's sire.

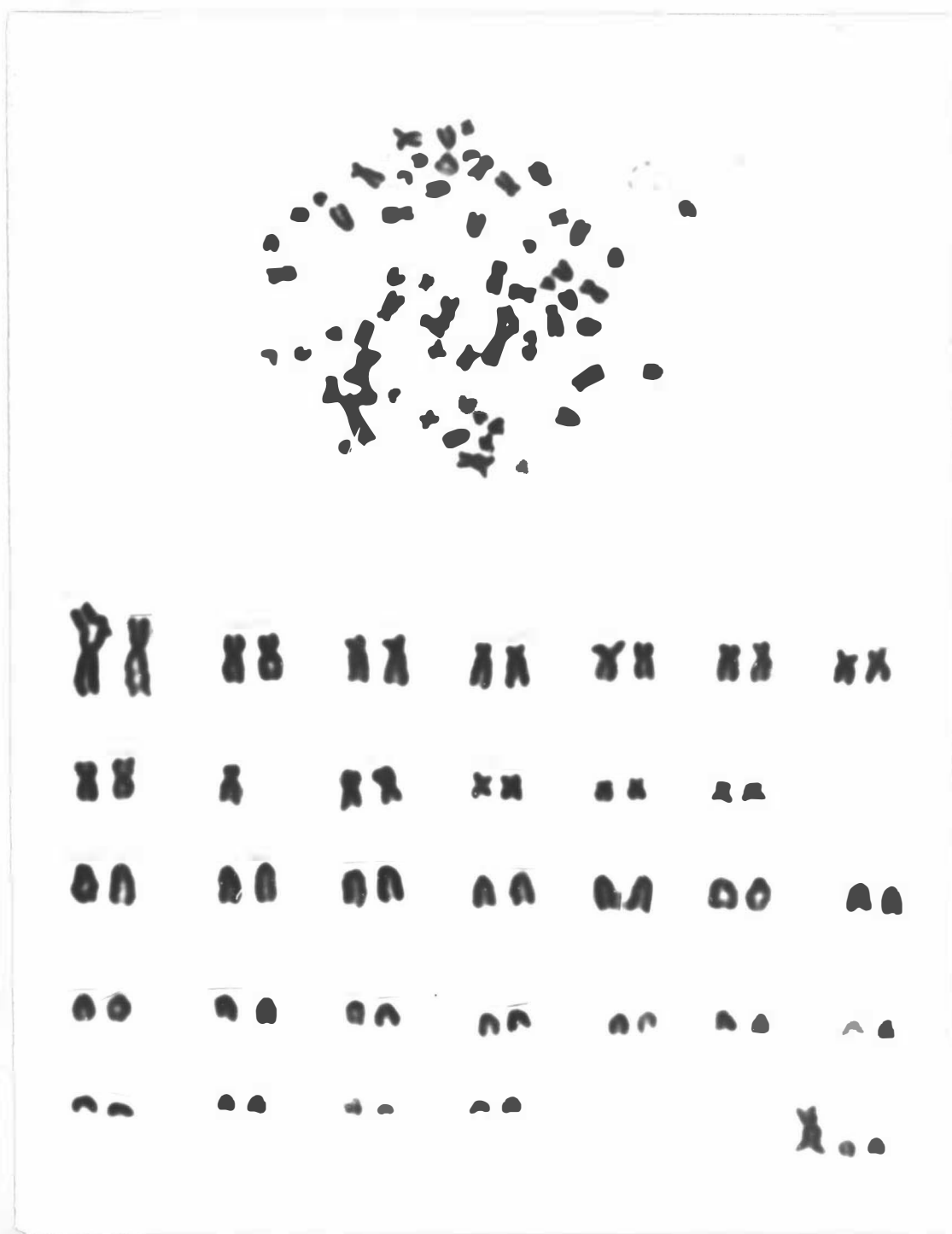
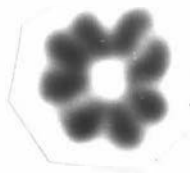


Figure III.8 Metaphase with 65 chromosomes and tri-radial (non-homologous chromatid interchange) involving chromosomes 1 and possibly 9. This was one of two cells with a chromosome count of 65 found in cultured cells from H-11's sire. (Approx. X 5,000)



- (a) A quadriradial chromosome arrangement involving either homologous or non-homologous chromatid interchange. Either two chromosomes from pairs 5 through 9 were involved, yielding a count of 64,XY, or a median sub-metacentric and two median acrocentric chromosomes, yielding a count of 65. (X 10,000)



- (b) Possible non-homologous chromatid interchange, probably involving a chromosome from one of the pairs 5 to 9 and pair 3. (X 5,000)

Figure III.9 Examples of abnormal chromosome arrangements found in (a) one of H-11's half brothers, H-41, and (b) H-11's 1978 colt, H-57.

It was thought that a mitotic instability might also have existed in H-41, one of H-11's half brothers. From each of H-11's half brothers evaluated, 42 cells were analysed. Two cells from H-41 had possible chromosome counts of 65. On table 3-3 both these cells are listed under counts of "other". In one of these cells it was equivocal on the basis of metaphase morphology whether two chromosomes indeed belonged to the metaphase. If they did the cell was 65/28 non-acrocentric, if they did not it was 63/27 non-acrocentric. The second ambiguous cell had poor chromosome morphology and contained the chromosome arrangement shown in Figure III.9a. If this arrangement contains a submetacentric and two acrocentric chromosomes the count became 65. Alternatively, if this arrangement contains two submetacentric chromosomes a normal 64/27 non-acrocentric male chromosome count resulted.

One of H-11's 1978 fillies, H-59, had two cells out of only 15 evaluated with possible chromosome counts of 65/28 non-acrocentric. These cells were listed under "other" on Table 3-3. In both cases the 'extra' chromosome was an acrocentric consistent with designation as one of the larger autosomes, although by metaphase morphology it was ambiguous whether it belonged to the metaphase. These cells raised the possibility of mosaic/chimaerism for a cell line containing 65 chromosomes, however, in one of H-11's fillies.

H-57, H-11's 1978 colt, had a "cold" at the time he was sampled. While his cells grew well in culture, there were many abnormal metaphases present. An example of an abnormal chromosome arrangement found in one of these (unanalysed) cells is presented in Figure III.9 b.

In the cells evaluated from H-68 there was no suggestion of a cell line other than 63,XO. The distinctive double heterochromatic band on the C-banded X-chromosome (Buckland *et al.*, 1976) confirmed that H-68 was an XO filly (Figure III.10). In addition, G-banded preparations (Figure III.11) confirmed the normalcy of H-68's autosomal complement, as well as the presence of a single X-chromosome. However, the possibility of another cell line being present to the degree of 6% was present at the 0.95 confidence level since 50 cells had been evaluated (Hook, 1977).

In the relatives and progeny of H-11, with the exception of H-68, the number of potentially 63,XO cells evaluated did not exceed

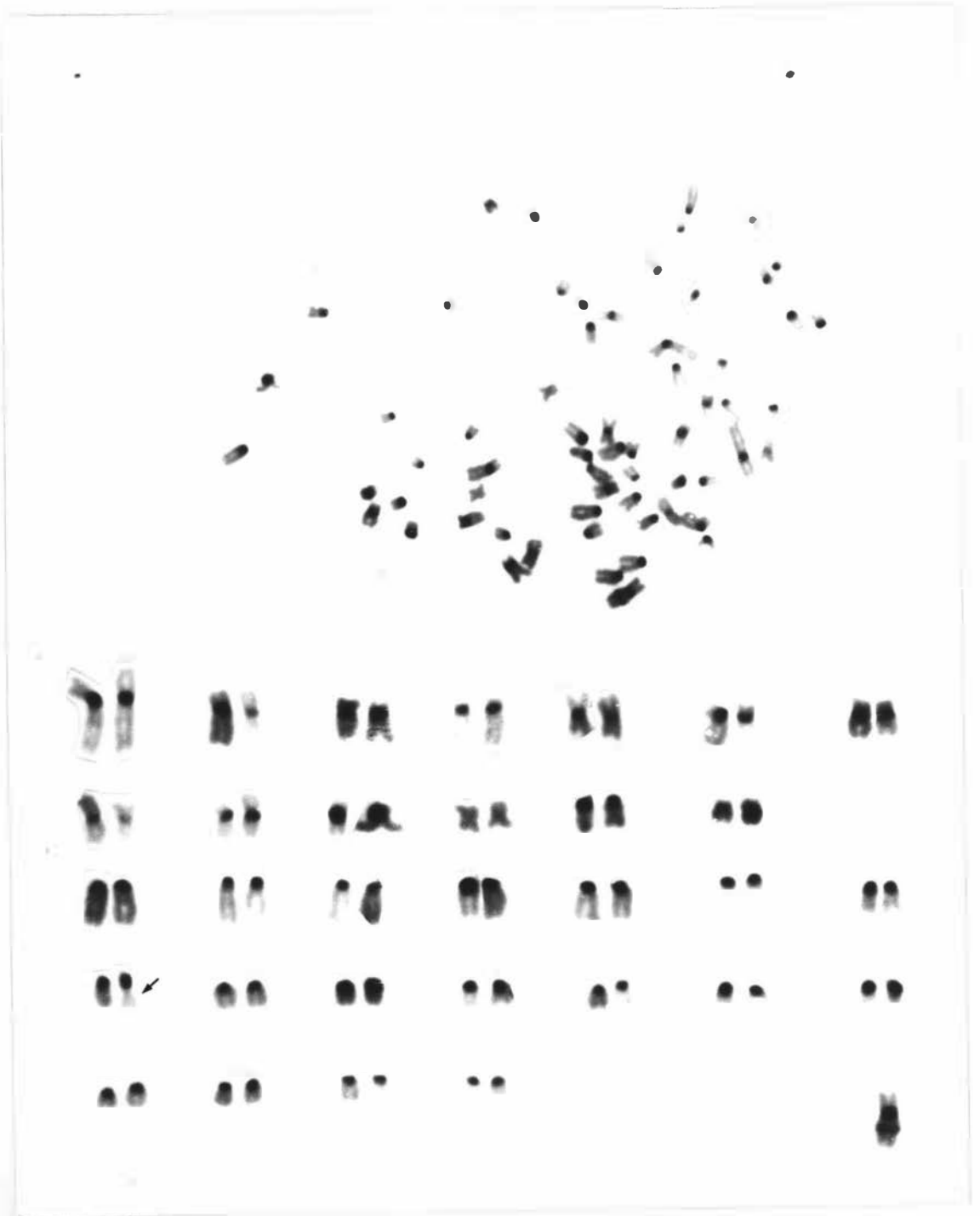


Figure III.10 C-banded cell from H-68, an XO filly sired by H-11. The characteristic X-chromosome's positive band on the long arm is distinct. Pair 13 displays the heteromorphic chromatin pattern, while one acrocentric (arrow) appears to have a chromatid break. (Approx. X 5,000)

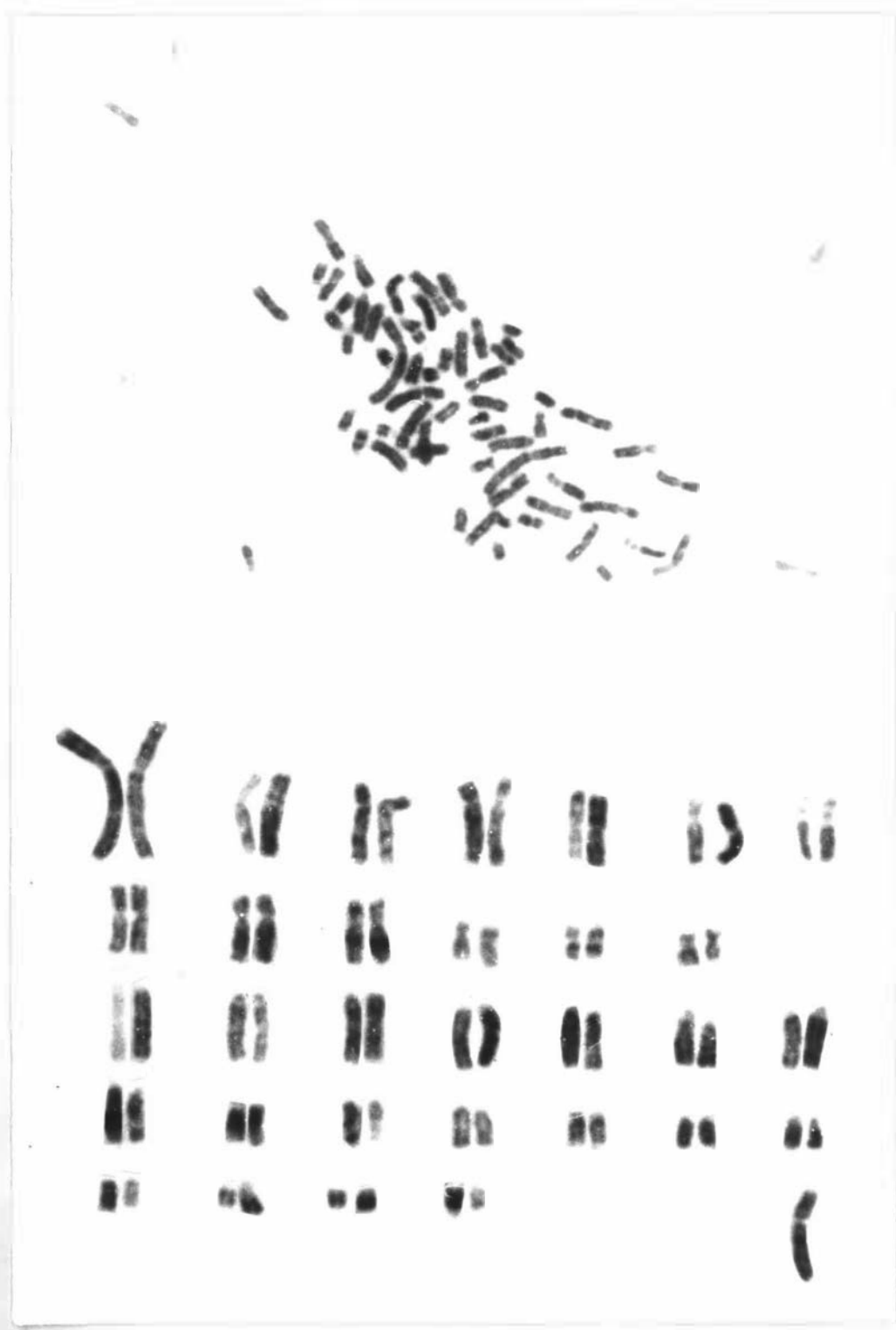


Figure III.11 G-banded cell from H-68, an XO filly sired by H-11.
(Approx. X 5,000)

the number of cells with estimated artificial chromosome loss. Therefore, the cells with counts of 63/27 non-acrocentric chromosomes were also considered to represent random loss.

On that basis, at the 0.95 confidence level, the degree of XO mosaicism that might have been present and gone undetected because of the numbers of cells evaluated in H-11's relatives and progeny (except H-68) were, according to Hook (1977): H-39, 5%; H-40 and H-41, 7%; H-57, 16%; and H-58 and H-59, 19%.

3.5. Discussion

There were at least five aspects of H-11's cytogenetic and genetic picture that deserve further comment: (1) his fertility, (2) that he sired an XO filly, (3) the abnormal chromosome arrangements found in cultured cells of his relatives and colt, (4) his size and behaviour in view of his XYY cell line, and (5) the possible manners of formation of his mosaic/chimaeric condition.

3.5.1. H-11's Fertility

There was little reason to suspect either possible infertility or sub-fertility in H-11 because of his chromosome constitution. While some impairment of spermatogenesis has been suggested in some human and murine XYY males, the reports are not consistent (Walzer *et al.*, 1978; Burgoyne, 1979). The majority of XYY and XY/XYY men are thought to be of normal fertility⁵, although some may be at risk of having abnormal children (LeMarec *et al.*, 1977). Though the sample was very small, there appeared to be no sex bias in H-11's progeny. An excess of progeny of one sex would indicate selection against a proportion of gametes (Dorus and Gillespie, 1978).

In fact H-11 appeared to be an above average sire. By the rules applied in calculating fertility rates for the Register of Thoroughbred Stallions of New Zealand, his rate was 100% (over 15 mares mated). In 1978 H-11 ran with the mares, but subsequently all (13) mares he mated were said to have conceived to first service. Furthermore, out of nine mares mated to H-11 which were capable of producing a live foal, eight had produced or were producing a live foal at term. This yielded a functional fertility rate of 88%. The average for a Thoroughbred stallion in New Zealand, based on reported figures in the Register of Thoroughbred Stallions of New Zealand, was between 50% and 60%.

⁵ What is normal fertility for a man, however, may be sub-fertility for a stallion. The criteria of measurable fertility in a man (progeny) is based on neither the quantity nor quality of individual attempts at impregnation, as it is in the stallion.

3.5.2. H-11's XO Filly

As a mosaic/chimaera H-11 may have been at risk of siring either chromosomally abnormal (Barr *et al.*, 1964; Dewhurst, 1978) or otherwise abnormal (LeMarec *et al.*, 1977) offspring. In cattle a familial tendency for non-disjunction in a sire's line associated with a high frequency of pathological conditions was identified by Rieck *et al.*, 1970; Rieck, 1974). Lajda *et al.* (1976) discovered a bull used for artificial insemination which was an XY/XXY/XO mixoploid. One of its offspring was an XO/XX mosaic, however 23% of its offspring had hypoplastic gonads and were infertile.

The discovery of an XO filly (H-68) by H-11 may have been fortuitous. Limited studies suggested that the XO condition was relatively common in the mare (Blue *et al.*, 1978; Walker and Bruère, 1979; Trommershausen-Smith *et al.*, 1979). However, H-68 was the first reported XO stillborn foal. H-68 was also the first known XO progeny of a known mosaic/chimaeric stallion, himself the first reported XY/XYY/?XO stallion. The dam of H-68 was not available for cytogenetic evaluation. She was 13 years of age at H-68's foaling, but as yet there is no evidence of a maternal age effect on the occurrence of the XO mare (see Chapter VII). The dam's reproductive history was incomplete, however, she had had at least four consecutive normal live foals prior to H-68, which made her an above average brood mare.

The cause of H-68's death was probably internal haemorrhage. Indirectly, however, the cause of death was most likely the foetal malposture which lead to the dystocia. Breech presentation is a relatively common obstetric complication in the horse, and it frequently leads to peri-natal death due to internal haemorrhage (Bruère, pers. comm.). Thus, as far as was known, it was only by chance that H-68 did not enter the population as another infertile mare, thereby reducing the breed's fertility.

However, there was no information on the incidence of chromosome anomalies in peri-natal equine mortalities. In humans chromosome abnormalities are frequently found in peri-natal and neonate mortalities (Alberman and Creasy, 1977; Sutherland *et al.*, 1978). The foetal mechanism that results in the breech presentation is unknown (Jeffcott and Rossdale, 1979), so it is not impossible that H-68's XO chromosome constitution may have contributed to her death. Further-

more, as no autopsy was carried out it was possible that H-68 may have had a major developmental defect, such as heart anomalies which are frequently found in new born animals (Bruère, pers. comm.) and XO infants (Singh and Carr, 1966), which may have also contributed to her death.

If there is a tendency for mosaic/chimaeras to sire cytogenetically abnormal offspring, the possibility that there are a number of fertile mosaic/chimaeras in the horse population would be suggested by the frequency of the XO condition in the domestic horse. The possibility of mosaic/chimaerism in H-11's sire (H-39) and one of H-11's fillies (H-59) was also considered, in view of H-68, suggestive of cytogenetic instability in mosaic/chimaeric horses.

3.5.3. The Abnormal Chromosome Arrangements in the Cultured Cells of H-11's family.

The abnormal chromosome arrangements seen in cells from H-11's family (Figures III.8 and III.9) are occasionally seen in cultured cells of domestic animals (e.g., Blue, 1976), however they have not been specifically studied in such species. In human cell cultures these abnormal chromosome arrangements occur rarely, and while they may relate to *in-vitro* conditions (P. Fitzgerald, pers. comm.) they are difficult to induce with high frequency or repeatability (Stahl-Maugé *et al.*, 1978; Mattei *et al.*, 1979). A chromatid break and an error in its repair must be postulated for their formation (Stahl-Maugé *et al.*, 1978).

In humans such abnormal chromosome arrangements, especially the tri-radials (Figures III.8, III.9b and possibly III.9a), are associated with specific fragile sites on chromosomes and are seen with increased frequency in situations of chromosomal instability and in familial aggregations (Shiraishi and Sandberg, 1977; Arlett and Lehmann, 1978; Stahl-Maugé *et al.*, 1978; Mattei *et al.*, 1979). Even in conditions and human families where these configurations are "frequently" found, they have been calculated to occur only in the order of four out of 10^4 cells evaluated (Stahl-Maugé *et al.*, 1978). If the frequency of occurrence were the same in horses as in humans, it is beyond probability that one such cell would be seen in each of three cultures during evaluation of less than 100 cells. Blue (1976)

found one such arrangement, involving chromosome pair number 11, in a 64,XY embryo during evaluation of 250 cells. So the question remained of possible fragile sites in the *E. c. caballus* karyotype. On the other hand, there may be a high frequency of chromatid breaks in certain horses, as in certain human families and conditions (Shiraishi and Sandberg, 1977; Arlett and Lehmann, 1978; Mattei *et al.*, 1979).

It was obvious, even in Figure III.9a, that all the chromosomes involved in these abnormal arrangements were not the same, although a medium-small sub-metacentric may have been involved in all three possible tri-radial examples. This raised the possibility of a familial abnormal chromosome(s). Abnormal chromosomes have been implicated in humans with a tendency toward mitotic and meiotic instability (Buchanan *et al.*, 1976; Ford and Lester, 1978; Fryns *et al.*, 1978; Heritage *et al.*, 1978; Karden *et al.*, 1980).

If an abnormal chromosome(s) was present in H-11's family, it would not, from the limited evidence of this study, seem to effect fertility. However, the possibility remained that an abnormal chromosome(s) may have been involved in the occurrence of an XO mare (H-68) whose sire was a mosaic/chimaera.

The most exciting aspect of the possibility of an abnormal chromosome(s) was its occurrence in a fertile animal who could be used in genetic research. This might be one key to tracing gene linkage and eventually to mapping equine chromosomes.

3.5.4. H-11's Size and Behaviour in View of His XYY line

3.5.4.1. H-11's size

H-11 was referred for cytogenetic analysis because he appeared to be unusually large for his age, when compared with his paternal half-sibs and other colts in that part of New Zealand. He was also considered to be large at birth.

From available evidence, H-11 was a low level XYY mosaic/chimaeric foal. Birth weight and length in XYY boys are within normal range (Owen, 1972; Ratcliffe, 1976; Walzer *et al.*, 1978). However, humans are born at an altricial (foetal) stage of development and horses are born at an precocial one, so any comparisons between these species should more properly be made at equivalent developmental

stages. The stage of development in horses during the last eight weeks of gestation would seem to be, in so far as rapid bone formation, hardening of tendons and ligaments, and strengthening of muscles (Axe, 1907; Barone and Bertin, 1966), about equivalent to a two to four year old child.

Height and rate of growth are commonly elevated in XYY boys, who normally experience a brief spurt of growth at some time between 2.5 and 4 years of age and another during adolescence (Daly, 1969; Ratcliffe, 1976). A growth spurt occurring during the equivalent periods in a horse could result in an unusually large foal. By the age of nine months H-11 had attained a size of 163.75 cm (16.1½ h.h.), an average height for a mature male of his breeding but above average for an "adolescent" colt. His subsequent rate of growth, based on generalized equine size for age data (Axe, 1907; Hintz et al., 1979) seemed to be normal.

H-11 was in size at the extreme end of the Thoroughbred height distribution (Willoughby, 1974). The majority of XYY men are also reported to be in the upper percentile of the human height distribution (Owen, 1972). However, by comparison, no abnormality of size was noted in either the XO/XYY or XYY mice reported (Cattanch and Pollard, 1969; Evans et al., 1969).

Thus, neither H-11's rate of growth nor attained size contradicts the possibility of an influence by his XYY cell line on his phenotype.

In terms of the genetics of phenotype in domestic animals, it is thought that gene systems which have a general effect on growth have a relatively strong influence at early ages (Johansson and Rendel, 1968). Group specific gene systems influencing skeletal growth and probably gene systems influencing specific traits such as height at withers, weight and body length, are thought to become more important as the animal develops (Johansson and Rendel, 1968).

In the Thoroughbred, heritability estimates are relatively low for the components of size of young foals, such as weight, height at withers and cannon bone circumference (Hintz et al., 1978). The components of mature stature in horses, on the other hand, have a high heritability, for example height at withers 0.44-0.88 (Kalmykov, 1975; Wrangmore, 1976; Hintz et al., 1978). The size of H-11's filly out of a pony mare, H-58, at nine months of age (156.25 cm or 15.2½

h.h.) suggested a strong genetic component to H-11's size. Although H-11's sire was a good sized Thoroughbred, he was not considered to be of exceptional size. However, H-11's dam may have contributed genes promoting large stature to him since his maternal half-brother, as a gelding, was reported to have attained a height at withers of 172.3 cm (17.1 h.h.).

H-11's rate of growth exceeded those of other pasture fed colts in his area. Post-weaning growth rate in domestic animals is sex influenced, but appears primarily a reflection of efficiency of feed conversion (Johansson and Rendel, 1968). However, it was considered doubtful that H-11's growth differential could be totally accounted for by superior feed conversion efficiency.

In horses there is a strong maternal influence on birth weight and size as shown by cross-bred foals (Johansson and Rendel, 1968; Jones and Bogart, 1971). How much maternal effects may have contributed to H-11's large birth size can not be known, other than that she was able to carry the foal to term. H-11 was nursed on a pony mare, but this should have had no marked effect upon his rate of growth. In all other respects his management was reported to have been normal for a Thoroughbred.

In summary, it was thought that H-11's rate of growth and attained size were unusual. It seemed possible that his XYY line may have influenced his rate of growth, allowing him to attain his genetically determined potential.

3.5.4.2. H-11's behaviour

XYY and XYY mosaic men have been suggested to be at high risk for aggressive and anti-social behaviour, sub-intelligence and weak gender identity or homosexual tendencies (Daly, 1969; Owen, 1972; Price et al., 1976; Bulrich et al., 1978; Walzer et al., 1978). The suggestion of such risks may either be solely sample bias or represent a primarily subjective culture or species specific evaluation (Hook, 1973; Hook, 1979). No non-humans with this chromosome constitution had been studied behaviourally. No "mental" or behavioural abnormalities were discovered in XXY sheep by Kilgour and Bruère (1970) or Bruère and Kilgour (1974). On the other hand, individual XO mares had been reported to be difficult to handle (Blue, 1976; anonymous, pers. comms.).

H-11 was a placid, good tempered stallion which was reported to have never shown aggressive tendencies. He had displayed no signs of abnormal sexual behaviour. According to Pickett et al. (1977) abnormal sexual behaviour commonly can occur in stallions which, like H-11, begin stud duties relatively young and have been treated roughly by a mare.

The various reports received on H-11's behaviour were consistent with an opinion that, with the possible exception of less than normal agonistic behaviour, he displayed normal (male) behaviour for his species, in so far as his restricted environment allowed.

3.5.5. Possible Manners of Formation of H-11's Mosaic/Chimaerism

The limitation of cells evaluated to those of mesodermal origin, limited as well speculation as to both the extent of H-11's mosaic/chimaerism and the possibility of whole body chimaerism. Haemopoietic chimaerism, however, could be excluded since evidence of an XYY cell line was found in both leucocytes and skin cultures.

H-11 was the first report of an equine (presumptive) XY/XYY/XO mixoploid. Without differential staining it is impossible to specifically identify the Y-chromosome in the horse. Since only conventional Giemsa stain was used on chromosome preparations from H-11, the 65,X line was assumed to be 65,XYY. This was both by convention and because abnormalities of the sex chromosomes are most consistent with viability (e.g., Boué and Boué, 1974). That there could have been a true mosaic 63,XO line followed from the most simple manner of formation of such a mixoploid.

The most simple manner of mosaic formation of H-11's mixoploidy was non-disjunction occurring in either a XY blastomere or XY primordial cell, creating minor XYY and XO cell lines (Ford, 1969), and postulating proliferative or selective advantage to the normal 64,XY cells.

It was interesting that all H-11's progeny, irrespective of the colouration of their dam, had been reported to have had four tall white socks. This raised the question of the possibility of genomic segregation having been involved in the non-disjunctional event that could have resulted in H-11's mosaic/chimaerism. Markings of the extremities of the horse are thought to be under polygenic determination (Castle, 1948; Jones and Bogart, 1971) and may be either

recessive or weakly dominant in like coloured horses (Jones and Bogart, 1971). It seemed unlikely that H-11 could have been homozygous and dominant at all loci for that trait unless some other factor were involved. No information was available on the facial markings of H-11's progeny, except by mare "B" which showed marked similarity to their sire (Figures III.2 and III.3b), and H-57 and H-59 which both had white face markings. Genomic segregation or some sort of homologous chromosome interchange involving multiple loci could be the more simple explanations for linked transmission of multiple alleles.

3.5.6. Summary

H-11 was the first report of a XY/XYY/?XO mosaic/chimaera. He was fully fertile, however, he sired an XO filly. This filly was the first reported stillborn XO mare. Limited cytogenetic investigations were also carried out on H-11's sire, two of his half-brothers and three of his other progeny. The results of these suggested that other cytogenetic irregularities might exist within the kinship.

CHAPTER IV
H-52, A MARE OF DISTINCTION

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CHAPTER IV

H-52, A MARE OF DISTINCTION

4.1. Introduction

H-52 was referred for cytogenetic analysis as a suspected XO mare. She was a seven year old Thoroughbred mare who was reported to have had two unsuccessful seasons at stud. Following evaluation of chromosome preparations from cultured lymphocytes, H-52 was tentatively diagnosed as a 64,XX/65,XXY mosaic/chimaera. A second blood sample was obtained to confirm that diagnosis. However, the second evaluation showed XX/XXX/XXY mosaic/chimaerism. H-52's owner donated her for further study. She arrived at Massey University on the 1st of March, 1979.

In view of H-52's unusual mixoploid mosaic/chimaerism, it was decided that she should be described in as many aspects as possible. However, some of these aspects, for example behaviour, had to be limited to descriptive and subjective observations.

It was considered significant that H-52 existed. Time and the investigation of other mares with her chromosome constitution(s) will determine the significance of both the cytogenetic findings and the observations in this Chapter.



Figure IV.1 Left lateral view of H-52.

4.2. Materials and Methods

4.2.1. Description

Sex: female
 Foaled: 1971
 Breed: Thoroughbred
 Colour: chestnut
 Size: 151.25 cm (15.½ h.h.) at withers
 (dimensions given in Appendix IIIa)
 Weight: 431.82 kilograms (at autopsy)
 Age of sire at birth: 10 years
 Age of dam at birth: 8 years
 H-52 is shown in the frontispiece and Figure IV.1.

4.2.2. History

H-52 was referred to the cytogenetics unit at Massey University in November, 1978, as a suspected XO mare. She was then seven years old, and was reported to have had two unsuccessful seasons at stud. Both her ovaries were said to be 1.5 cm in size. In January, 1979, it was reported that she had come into oestrus and a 2.0 cm follicle was palpated per rectum by her veterinarian. At that time her left ovary was said to be 2.5 cm in length and elongated and the right ovary 1.25 cm and round. She was not served since it was then too late for the mare to foal within the acceptable period for Thoroughbreds.

4.2.3. Cytogenetic Materials and Methods

4.2.3.1. Cell Cultures

Lymphocytes were cultured as described in Appendix VI. Over fifteen separate lymphocyte cultures were made from blood from H-52. From each of four lymphocyte cultures 30 or more cells were evaluated. From eight of the other cultures a total of 55 cells were also evaluated.

Solid tissues were cultured using the procedures outlined in Appendix VI. Table 4-1 gives the tissues, the technique used per culture set (individual culture flasks), and the minimum and maximum

Table 4-1

Summary of cell culture techniques and culture periods of non-lymphocyte cultured cells.

TISSUE (flask)	TECHNIQUE	DAYS IN CULTURE	
		MINIMUM	MAXIMUM
Skin I-s (1-4)	plasma clot	10	48
Skin I-ss (1-4)	plasma clot	10	24
Uterus II (1-6)	plasma clot	28	63
Skin II (1) (2) (3) (4)	plasma clot	11	32
	plasma clot	30	120
	plasma clot	44	50
	plasma clot	30	30
Peritoneum I (1) (2) (3) (4)	plasma clot	24	24
	plasma clot	20	46
	plasma clot	18	30
	plasma clot	18	26
Uterus III (1) (2) (3) (5)	plasma clot	30	76
	plasma clot	46	46
	plasma clot	76	76
	plasma clot	46	76
Left ovary (2) (3) (4)	plasma clot	20	43
	plasma clot	25	36
	plasma clot	25	34
Right ovary (1) (3)	plasma clot	22	34
	plasma clot	25	43
Right cornea (1) (2)	plasma clot	12	30
	plasma clot	12	16
Left cornea (1) (2)	plasma clot	12	26
	plasma clot	12	16
Right retina (1) (2)	plasma clot	20	40
	plasma clot	17	37
Left retina (1) (2)	plasma clot	27	30
	plasma clot	28	37
Broad ligament (1) (2) (3)	plasma clot	37	37
	plasma clot	20	20
	plasma clot	27	27
Intestinal mesentery (1) (2) (3)&(4)	plasma clot	27	37
	plasma clot	28	42
	plasma clot	27	40
Kidney (3) (4)	trypsin	20	20
	trypsin	25	25
Lung (4)	plasma clot	25	40
Thyroid (1) (4)	trypsin	20	20
	plasma clot	30	66
Parathyroid (1) (2)	plasma clot	20	50
	plasma clot	17	33
Peritoneum II (2) (3)	trypsin	30	40
	plasma clot	40	42
Spleen (1)&(2) (3) (4)	trypsin	20	20
	plasma clot	27	48
	plasma clot	40	40
Smooth muscle (duodenum) (1)	plasma clot	20	50

Note: Chromosome preparations from culture with minimum culture times were analysed. However, no culture effect on proportions of cell types present was noted, with possible exception of uterus II.

culture period for each set. Cells used for chromosome analysis, from all tissues evaluated, had the minimum culture time. Sets of cell cultures were analysed separately, however there was no difference in the proportions of the cell types present between sets.

No attempt was made to separate component tissue types (e.g., epithelium, endothelium, vascular elements) except in Skin I, where an attempt was made to dissect epidermis (Skin I-s) from dermis (Skin I-ss). By cellular morphology in-vitro, the corneal cell cultures were predominately endothelial, Uterus II almost exclusively epithelial-like and Uterus III, kidney and thyroid cell cultures showed a mixture of epithelial-like and fibroblast-like cells.

The Skin I cultures were initiated from a skin biopsy taken under local anaesthesia from the left foreshoulder and Skin II was obtained from the abdomen during an exploratory laparotomy. Peritoneum I and Uterus III were also obtained during that laparotomy. Uterus II culture was initiated from an endometrial biopsy. All other cell cultures were initiated from tissues obtained at autopsy.

The majority of H-52's cells had a diploid count of 65 chromosomes (62 autosomes and 3 sex-chromosomes). As 65 was the modal number, standard cytogenetic terms have been placed within single quotation marks when they refer to the usual $2N = 64$ for the horse.

To estimate the amount of 'pseudodiploidy' (64,XX cells present through artifact), the percentage of cells evaluated per tissue which had chromosome counts of ≈ 62 , 63(28), 63(29) and 64(29) was used. The number in parenthesis represents the differential non-acrocentric chromosome count. Cells with differential chromosome counts of 64(28) in excess of this estimated rate of artifactual aneuploidy were considered to represent true 'diploid' cells.

4.2.3.2. Sex-chromatin Investigations

Sex chromatin investigations were carried out as is described in Appendix VI.

4.2.4. Examinations and Observations

Veterinary aspects of H-52 were investigated by members of the Department of Clinical Sciences, Massey University.

Opinions and advice was sought from persons with experience with normal horses. Some aspects of H-52's behaviour were brought to the attention of the author, others were noticed during personal involvement with the mare.

The author had close daily association with H-52 while the mare was at Massey University. This permitted continuity of observation and trained one person to recognize some subtleties of H-52's behaviour. On the other hand, the relationship that developed as a result of that daily contact clearly influenced some of the mare's reactions when the author was present. However, for a good part of H-52's stay she was in a paddock which could be observed over extended periods from the 7th floor tissue culture laboratory of the Veterinary Tower.

The author, as primary observer, was constantly aware of the probable bias introduced both by the relationship between observer and the mare, and by personal unfamiliarity with equine behaviour. Therefore, whenever possible personal observations and opinions were checked by other people.

4.3. Results

4.3.1. Results of Cytogenetic Investigations

Tissues from H-52 which were examined for mosaic/chimaerism, using cell culture techniques, are given below according to the principle presumptive embryonic germ layer from which they arose (Arey, 1962; Duke, 1970; Marrable, 1971; Guillemin, 1977).

<u>Ectoderm</u>	<u>Mesoderm</u>
retina (left)	kidney
retina (right)	spleen
cornea (left)	T-lymphocytes
cornea (right)	smooth muscle of duodenum
skin (epidermis)	intestinal mesentery
parathyroid (upper)	peritoneum
	ovary (left)
<u>Entoderm</u>	ovary (right)
thyroid	broad ligament
lung	uterus
	skin (dermis)

A total of 947 metaphases were evaluated from H-52 (Table 4-2). Four-hundred and ninety-four of these (52.2%) contained 65 chromosomes. The cells with 65 chromosomes had differential counts either of 28 non-acrocentric chromosomes (Figure IV.2) which were confirmed by G-banding to be 65,XXY cells (Figure IV.3), or 29 non-acrocentric chromosomes (Figure IV.4) which were confirmed by G-banding to be 65,XXX cells (Figure IV.5). There was the suggestion of a minor 64,XX cell line in both leucocyte cultures and one skin culture (Figures IV.6 and IV.7). Furthermore, there was evidence of a line or lines containing 66 chromosomes in non-leucocyte cultures. On the basis of chromosome morphology these cells were found to display either 66,XXX (Figures IV.8 and IV.9) or, in three cases, 66,XXXX (Figures IV.10 and IV.11). No suggestion was found of either a 63,XO or 64,XY cell line.

In addition 897 neurons from H-52's cerebellum and spinal cord (Ectodermal germ layer) were examined for sex-chromatin. Some cells were found with two sex-chromatin bodies (Figure IV.12a) which confirmed the presence of two inactive X-chromosomes in those neurons. A few cells were found with three sex-chromatin bodies, confirming the presence of three inactive X-chromosomes in some neurons. There was no suggestion of either an XO or XY cell line in the neurons examined.

Table 4-2

The distribution of gross chromosome counts from H-52's cultured cells.

TISSUES	DISTRIBUTION OF GROSS COUNTS						TOTAL CELLS
	≤ 62	63	64	65	> 65	Polyploid*	
Lymphocytes	18	24	77	78	1	2	200
(%)	(9.0)	(12.0)	(38.5)	(39.0)	(0.5)	(1.0)	
Solid Tissues	92	53	116	416	21	49	747
(%)	(12.3)	(7.1)	(15.5)	(55.7)	(2.8)	(6.6)	
TOTAL	110	77	193	494	22	51	947
(%)	(11.6)	(8.1)	(20.4)	(52.2)	(2.3)	(5.4)	

*Polyploid cells were recorded only if they could be karyotyped.

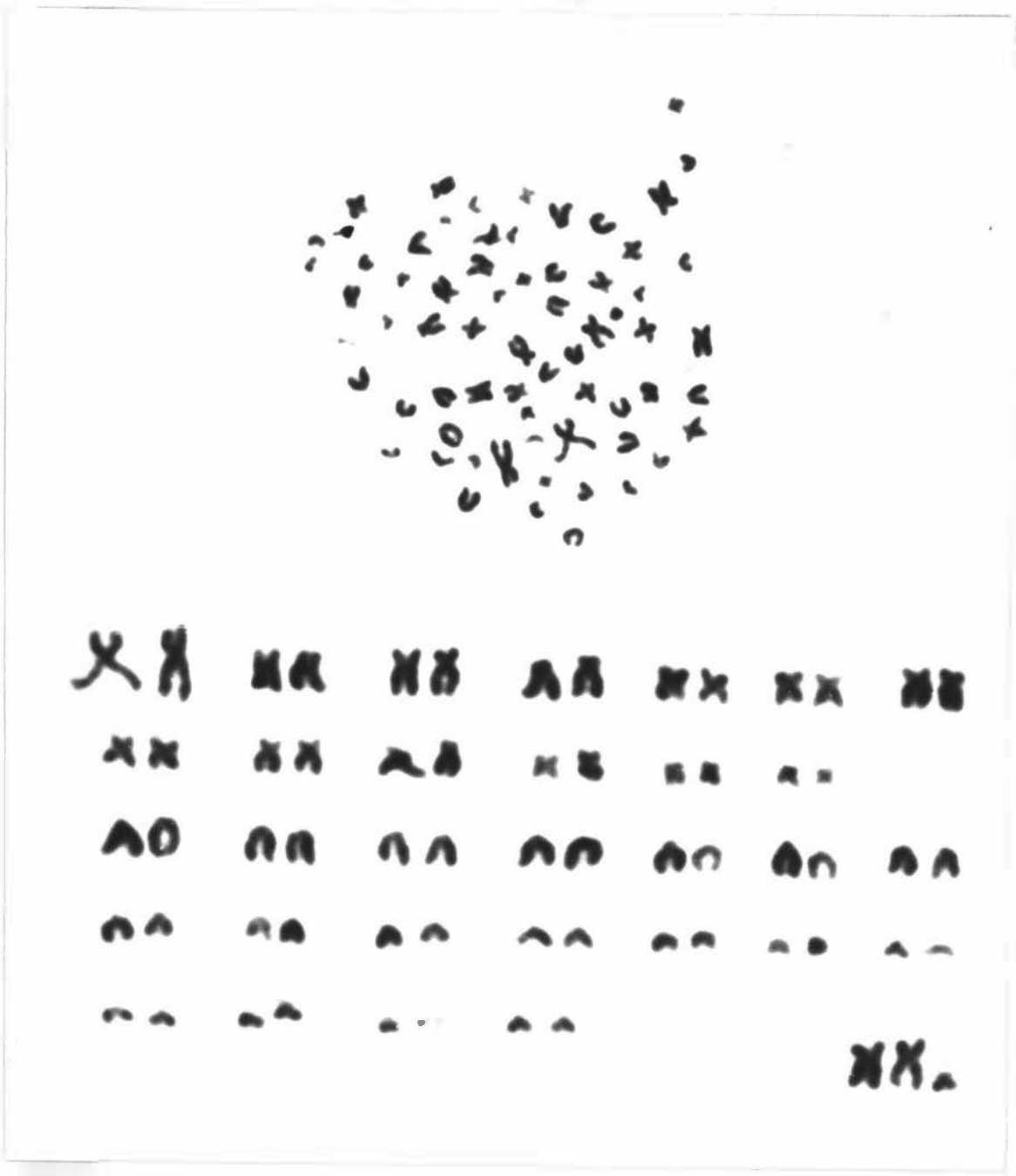


Figure IV.2 A metaphase containing 65 chromosomes, 28 non-acrocentric (approx. X 5,000). By convention the 37th acrocentric was designated a "Y" chromosome.

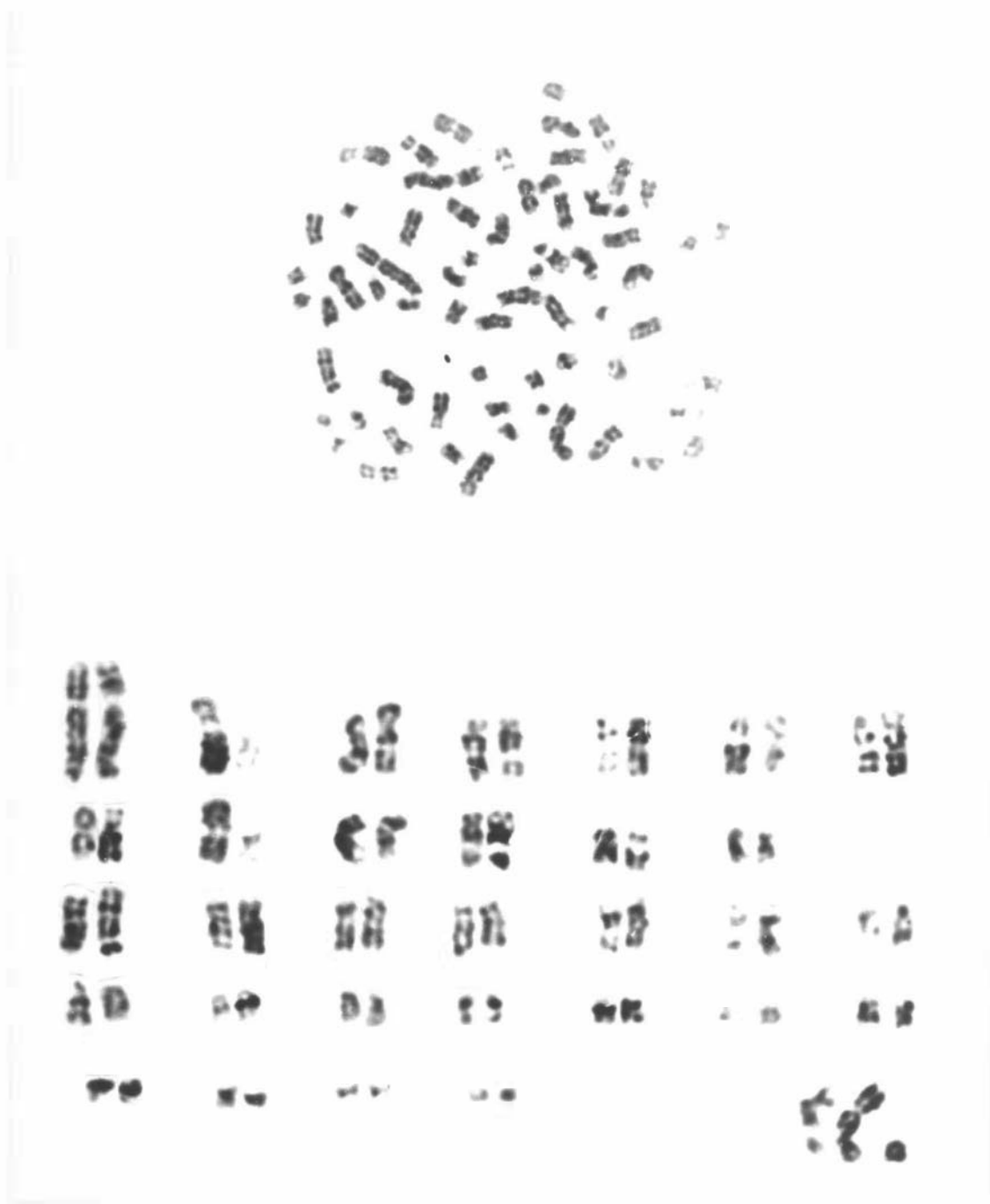


Figure IV.3 G-banded metaphase containing 65 chromosomes, 28 non-acrocentric, confirming the 65,XXY cell line (approx. X 5,000).

1001

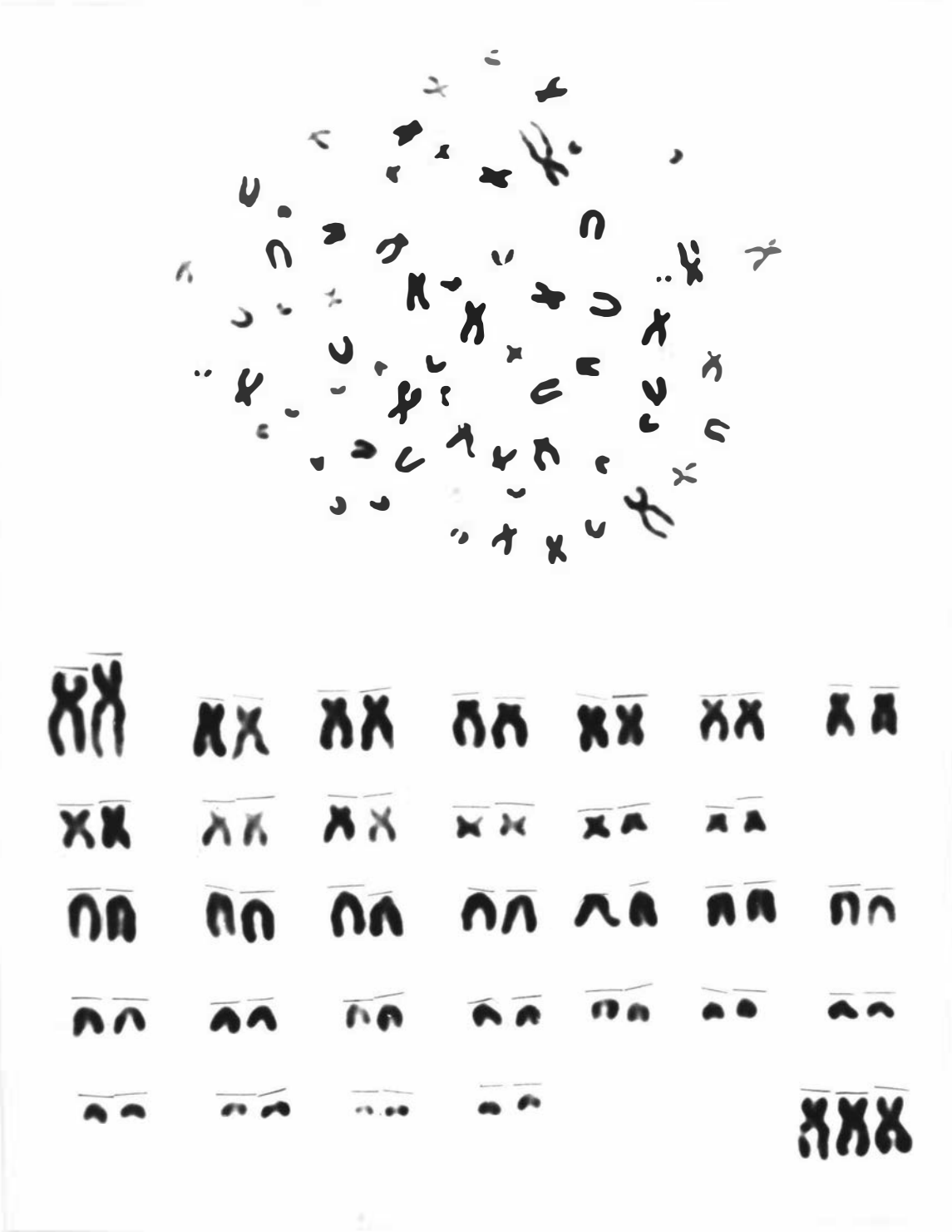


Figure IV.4 A metaphase with 65 chromosomes, 29 non-acrocentric, designated 65,XXX on the basis of chromosome morphology (approx. X 5,000).

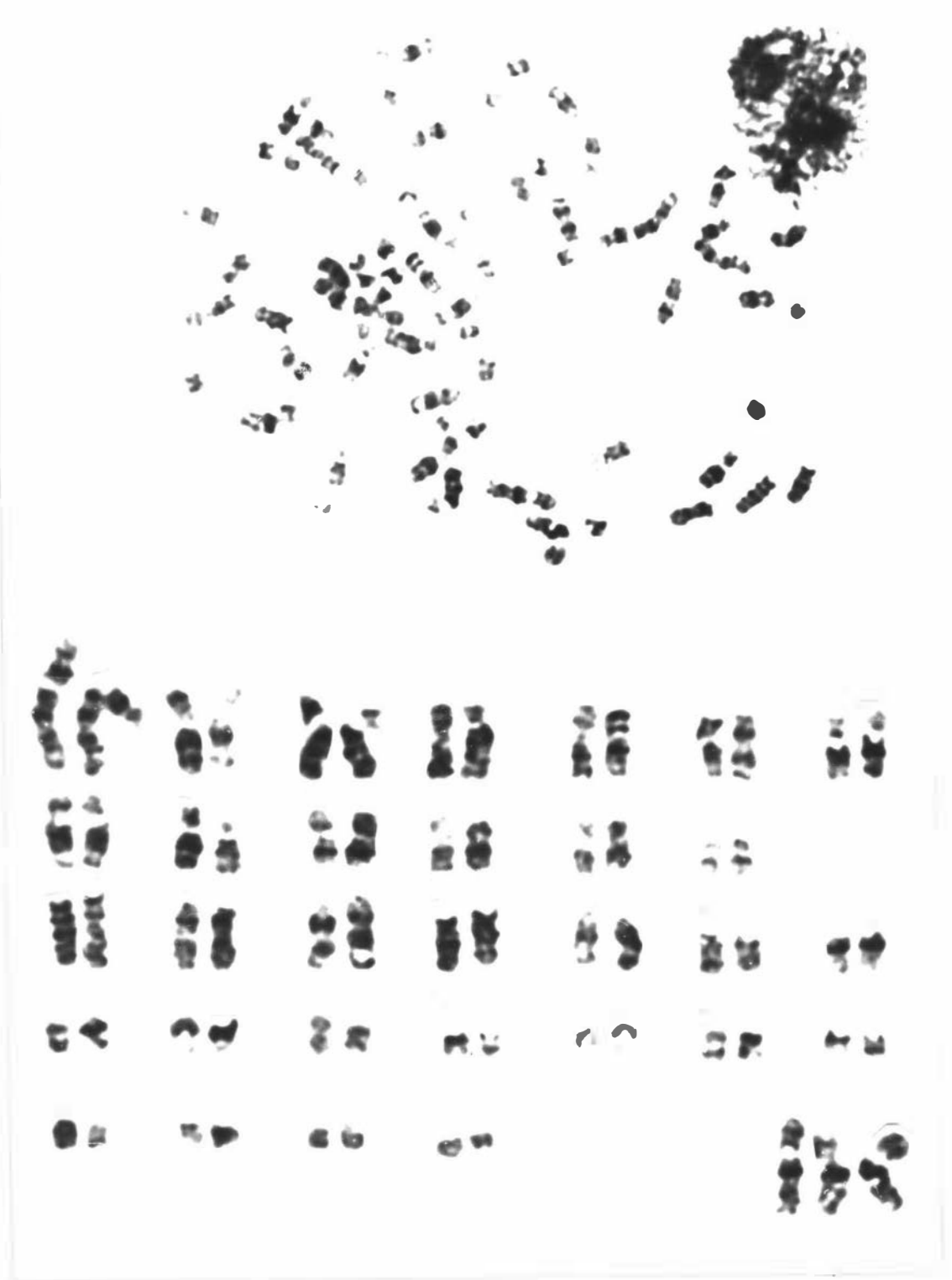


Figure IV.5 G-banded metaphase containing 65 chromosomes, 29 non-acrocentric, confirming the 65,XXX cell line (approx. X 5,000).

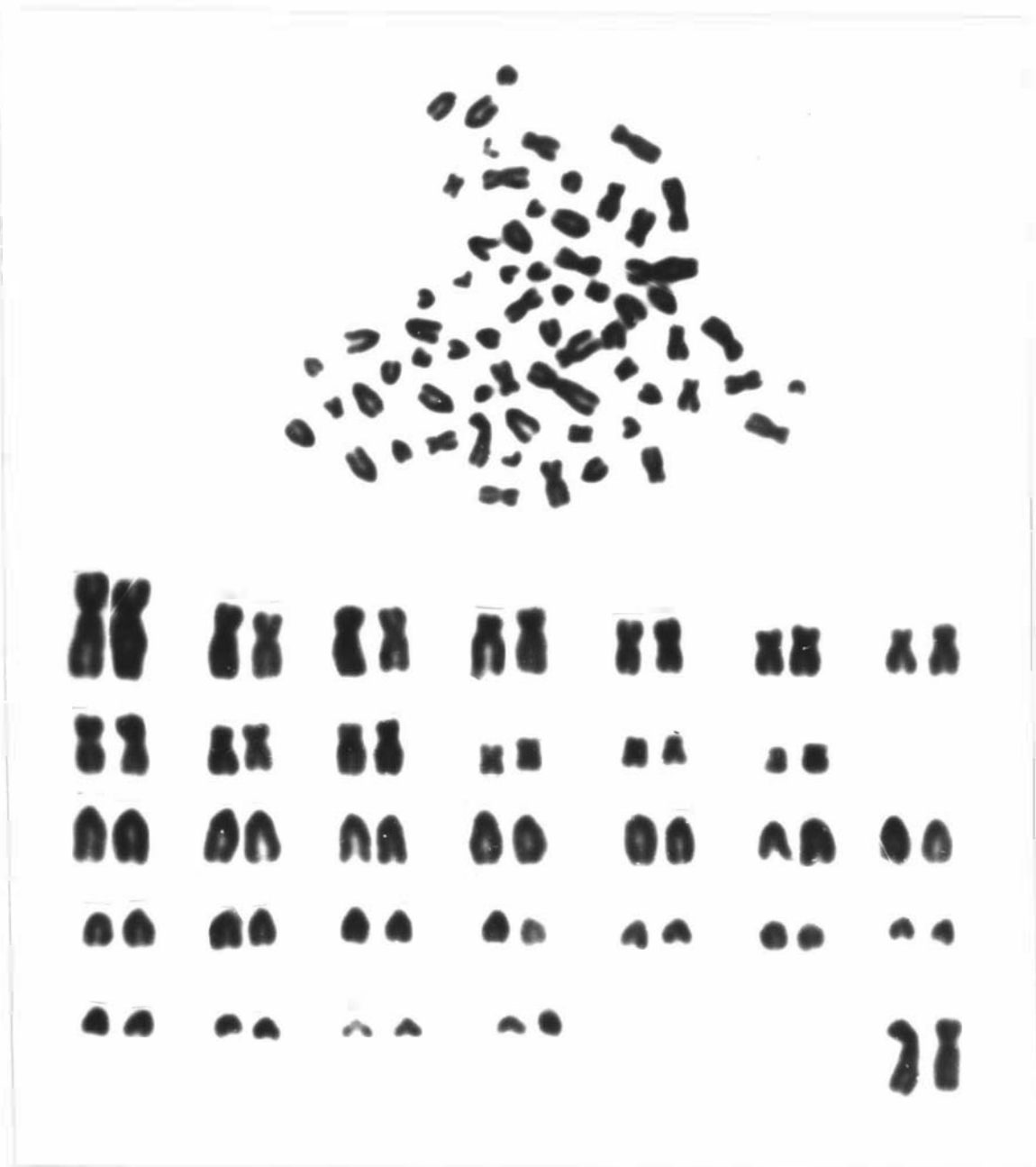


Figure IV.6 A metaphase with 64 chromosomes (28 non-acrocentric) containing two X-chromosomes (approx. X 5,000). This cell may represent either a 64,XX cell or one with 65 chromosomes that has lost a chromosome through artifact.

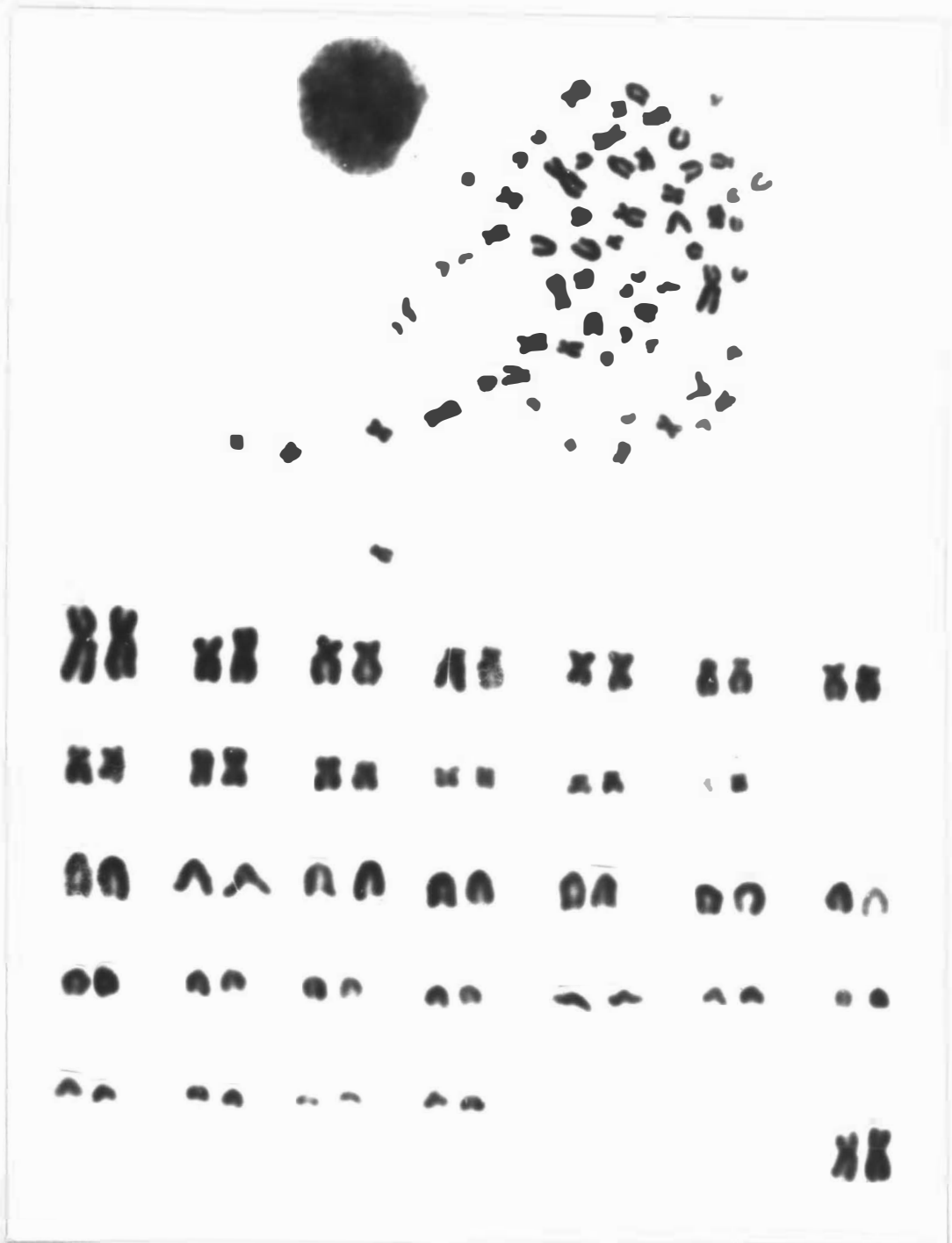


Figure IV.7

Another example of a 64,XX cell from H-52
(approx. X 5,000).

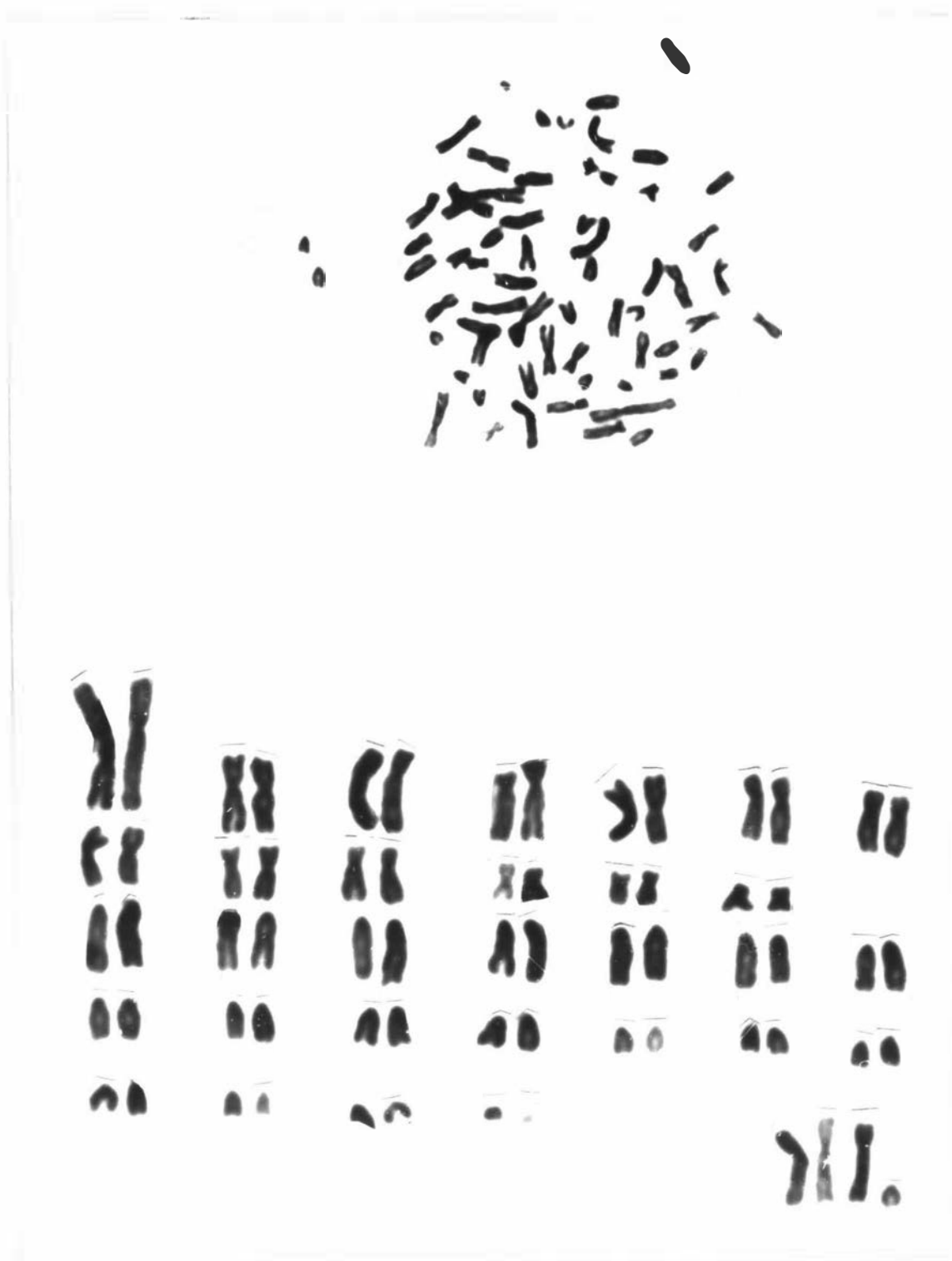


Figure IV.8 One of the metaphases with 66 chromosomes (29 non-acrocentric)(approx. X 5,000). On the basis of chromosome morphology and by convention this cell was designated 66,XXXY.



Figure IV.9 Another metaphase with 66 chromosomes
 (29 non-acrocentric) designated 66,XXX^Y
 (approx. X 5,000).



Figure IV.10 One of three metaphases with 66 chromosomes (30 non-acrocentric)(approx. X 5,000). This cell was designated 66,XXXX on the basis of chromosome morphology. Confirmation of this designation was obtained from sex-chromatin studies.

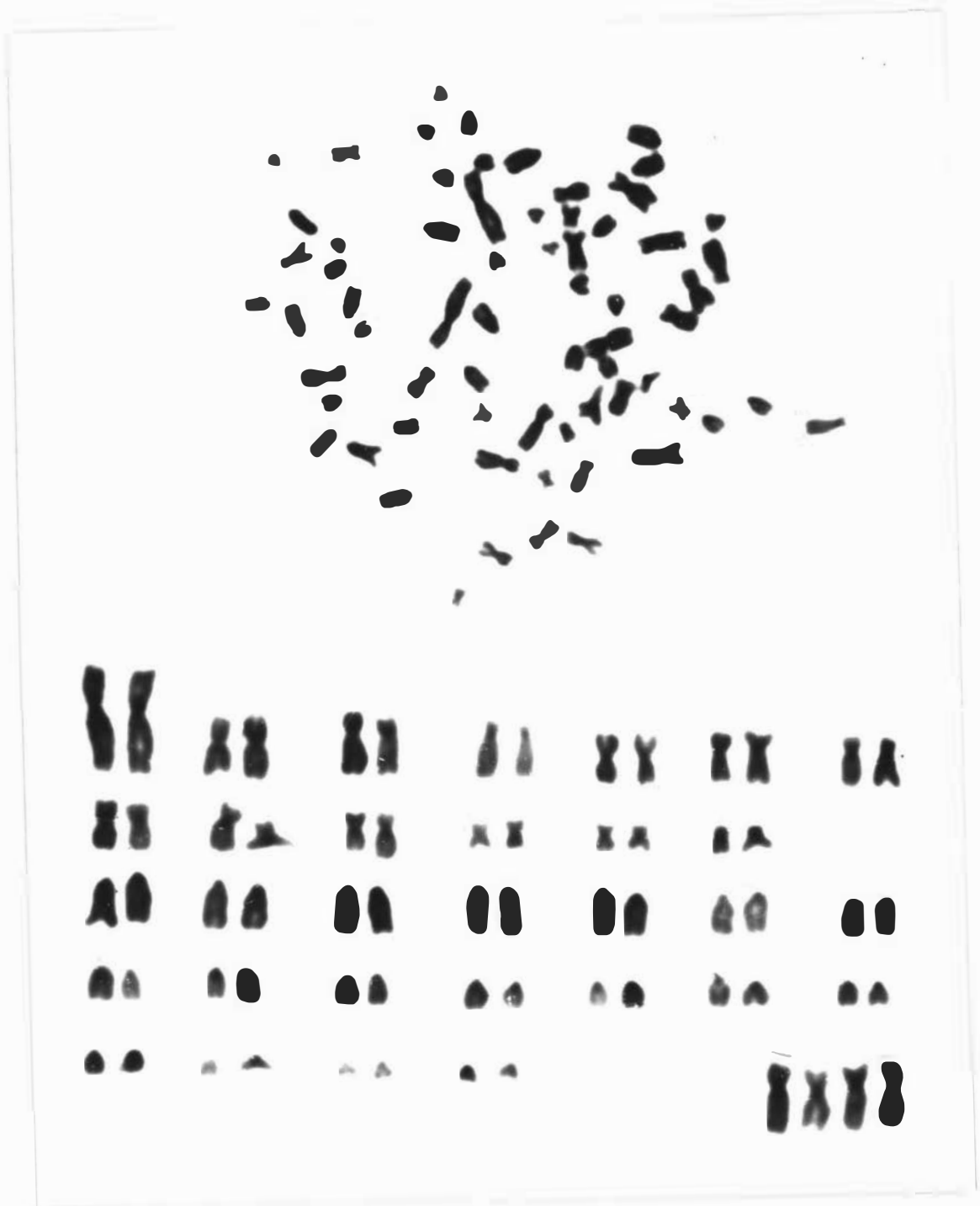


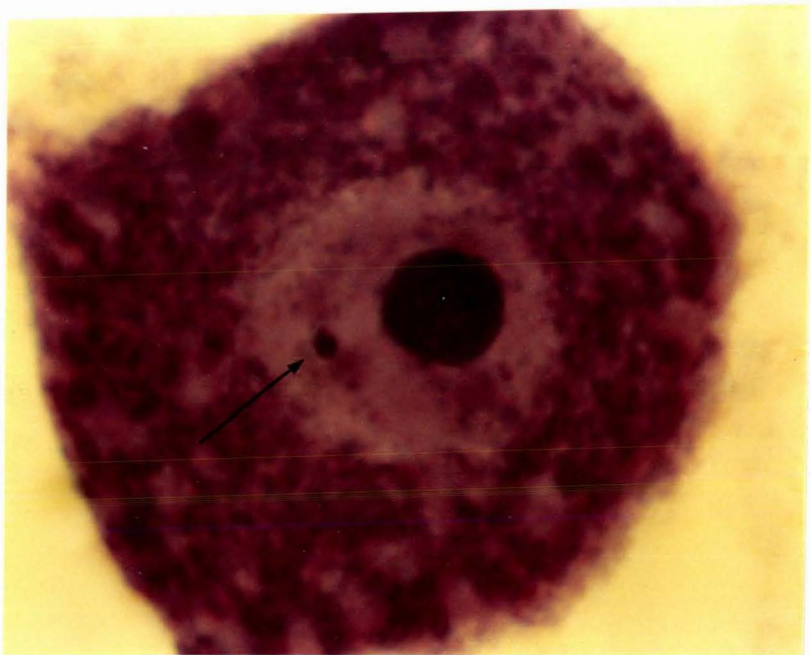
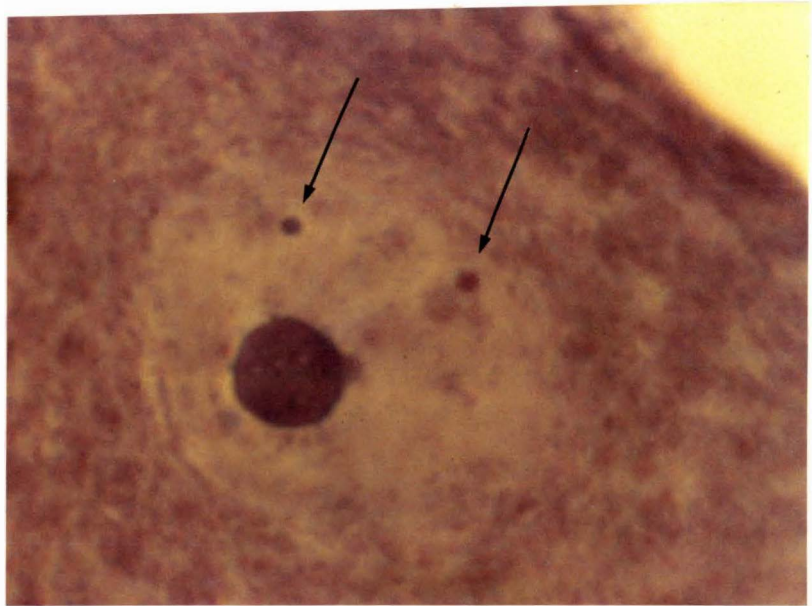
Figure IV.11 Another of the three metaphases designated 66,XXXX (approx. X 5,000).

Figure IV.12 a

An example of a neuron with two sex-chromatin bodies, in the nucleoplasm, confirming the presence of either XXX or XXXY cells (X 3,500).

Figure IV.12b

An example of a neuron with one sex-chromatin body in the nucleoplasm, which could represent either a XX or XXY cell (X 3,500).



4.3.1.1. Results of lymphocyte cultures

Results of chromosome counts from cultured lymphocytes are given as gross (un-differentiated) counts in Table 4-3, and partitioned by the differential non-acrocentric chromosome count in Table 4-4.

An histogram representing the proportions of the cells counted with 64 and 65 chromosomes, which by their differential count had 64,XX, 65,XXY and 65,XXX karyotypes, is presented in Figure IV.13. No adjustment was made for possible artifactual 64,XX cells when calculating the proportions shown in Figure IV.13.

Overall the estimated rate of artifactual aneuploidy was 24%. However, since the major component of that rate was from one culture (number II), a 64,XX line was believed to have existed in H-52's lymphocytes. Only one cell with 63/27 non-acrocentric chromosomes was found. This cell might have been 63,XO, but since only one was found it was considered to be an artifact.

Table 4-3

The distribution of gross chromosome counts in H-52's cultured lymphocytes.

CULTURE NUMBER	DISTRIBUTION OF GROSS COUNTS						TOTAL CELLS
	≤ 62	63	64	65	$>65^*$	Polyploid**	
II	5	12	13	13	1	-	44
III	2	4	12	16	-	1	35
IV	-	-	2	2	-	-	4
VI	3	3	15	12	-	1	34
IX	2	2	13	15	-	-	32
X	-	-	4	4	-	-	8
XI	3	-	2	1	-	-	6
XII	1	2	4	1	-	-	8
XIII	-	1	2	2	-	-	5
XIV	-	-	6	4	-	-	10
XV	1	-	2	7	-	-	10
XVI	1	-	2	1	-	-	4
Total	18	24	77	78	1	2	200
(%)	(9.0)	(12.0)	(38.5)	(39.0)	(0.5)	(1.0)	

* Part of a polyploid cell.
 ** Polyploid cells were only recorded when they could be karyotyped.

Table 4-4

Condensed distributions of differential chromosome counts from H-52's cultured lymphocytes.

CULTURES	DISTRIBUTION OF DIFFERENTIAL COUNTS										TOTAL CELLS
	≤62	63			64		65		>65	P*	
		(28)	(27)	(29)	(29)	(28)	(29)	(28)	(-)		
Blood II	5	10	2**	-	-	13	1	12	1	-	44
Blood III	2	4	-	-	1	11	8	8	-	1	35
Blood VI	3	3	-	-	-	15	9	3	-	1	34
Blood IX	2	2	-	-	2	11	7	8	-	-	32
Others***	6	2	1	-	3	21	17	5	-	-	55
TOTAL	18	21	3	0	6	71	42	36	1	2	200
(%)	(9.0)	(10.5)	(1.5)	(0)	(3.0)	(35.5)	(21.0)	(18.0)	(0.5)	(1.0)	

"Broken cells" = 24%

*P = polyploids. Cells were recorded only when they could be karyotyped.
 ** These were 63,XX cells
 *** Combination of counts from cultures where less than 30 cells were evaluated.

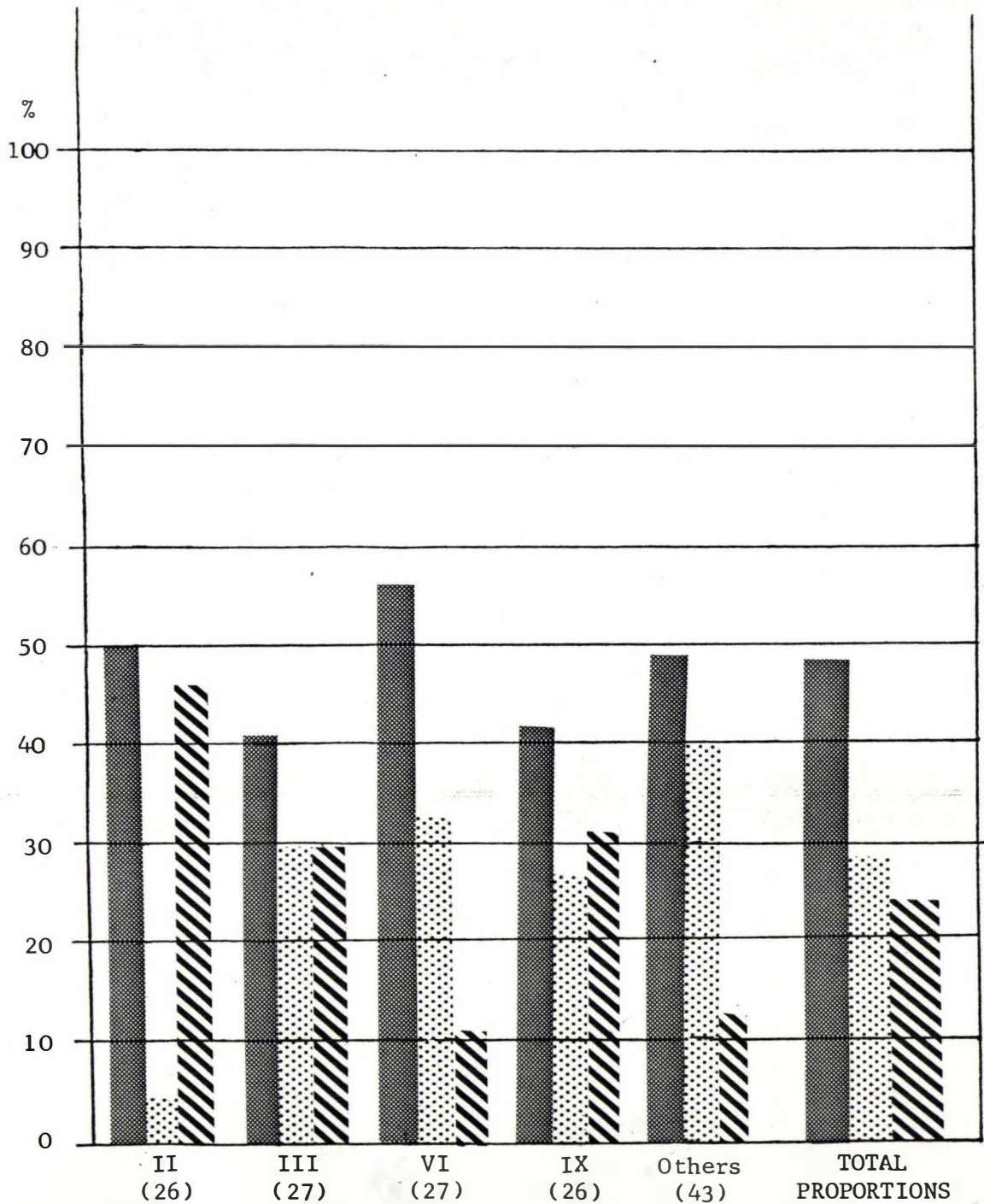


Figure IV.13

Histogram of cultured lymphocytes analysed with (gross) counts of either 65 or 64 chromosomes, showing the proportions (%) of XX, XXX and XXY cells.

■ = 64,XX, ● = 65,XXX and ▨ = 65,XXY.

4.3.1.2. Results of cultured cells (other than lymphocytes)

The gross distribution of chromosome counts from cultured cells other than lymphocytes are presented in Table 4-5. The differential (non-acrocentric) chromosome count distribution is shown in Table 4.6.

Based on the karyotype of the normal 64,XX mare, the average estimated rate of artifactual aneuploidy was 25.6%, with a range of 8.33% (Skin I-ss) to 43.2% (left retina). The average percentage of 64,XX cells (by differential count of 28 non-acrocentric chromosomes) was 9.24%, with a range of 0 (smooth muscle) to 46.1% (uterus II). However, in H-52 a 64,XX cell could be a normal cell or it could represent either a 65,XXX or a 65,XXY cell that had lost either an X-chromosome or an acrocentric chromosome respectively.

Cells with chromosome counts of 64/28 non-acrocentric in excess of the estimated tissue specific rate of artifactual chromosome loss were considered indicative of the possible degree of a true 64,XX cell line. By that criteria (Table 4-7) Skin I-s, Skin I-ss, and Uterus II raised the possibility of a true 64,XX cell line. Estimates of the degree of possible mosaic/chimaerism for a 64,XX cell line in these tissues were: Skin I-s, approximately 8%; Skin I-ss, 10-33.3%; and Uterus II, 20.5-26.9%.

Skin I-s's estimated degree of 64,XX mosaic/chimaerism was below both the tissue specific and the average rate of estimated artifactual aneuploidy, so an in-vitro clone effect might have been suspected in that culture. Since mosaic/chimaeric compositions can vary from area to area of the same tissue, the lack of suggestion of 64,XX mosaic/chimaerism in Skin II and Uterus III cell cultures could not be considered to reflect more than the composition of the explants that grew in culture.

There was no suggestion of a 63,XO cell line. Ten of the cells with a chromosome count of 63/27 non-acrocentric which might have been 63,XO cells were considered to be artifacts, since they appeared in cultures at a rate well below the estimated rate of artifactual aneuploidy.

Twenty-one cells evaluated (2.8%) showed hyperdiploid¹ chromosome numbers. Three (0.4%) were clearly parts of polyploid

¹In reference to H-52, hyperdiploid is used to denote cells with more than 65 chromosomes.

Table 4-5

The distributions of gross chromosome counts in cultured cells other than lymphocytes.

TISSUES	DISTRIBUTION OF GROSS COUNTS						TOTAL CELLS
	≤ 62	63	64	65	> 65*	Polyploid**	
Skin I-ss	1	1	10	11	-	1	24
Skin I-s	4	1	8	15	3	6	37
Skin II	2	-	4	22	2	1	31
Peritoneum I	4	2	4	26	-	1	37
Peritoneum II	5	3	2	20	-	2	32
Uterus II	3	2	12	9	-	-	26
Uterus III	3	2	1	28	-	1	35
Right cornea	6	3	9	19	3	1	41
Left cornea	6	2	7	23	4	6	48
Right retina	7	5	10	12	-	3	37
Left retina	4	4	9	12	-	2	31
Parathyroid (upper)	6	7	9	34	1	5	62
Thyroid	4	2	5	15	1	3	30
Kidney	12	1	7	30	2	8	61
Left ovary	8	3	3	28	-	-	42
Right ovary	4	-	4	24	-	1	33
Spleen	5	2	2	20	-	2	31
Broad ligament	4	3	4	18	-	1	30
Intestinal mesentery	2	4	4	17	4	5	36
Lung	2	1	2	26	1	-	32
Smooth muscle (duodenum)	-	4	-	7	-	-	11
TOTAL	92	53	116	416	21	49	747
(%)	(12.3)	(7.1)	(15.5)	(55.7)	(2.8)	(6.6)	

* See text.
** Polyploid cells were only recorded when they could be karyotyped.

Table 4-6

The distributions of differential chromosome counts in cultured cells other than lymphocytes.

TISSUES	DISTRIBUTION OF DIFFERENTIAL COUNTS										TOTAL CELLS
	≤ 62	63			64		65		>65	P*	
		(28)	(27)	(29)	(29)	(28)	(29)	(28)	(**)		
Skin I-ss	1	-	1	-	-	10	10	1	-	1	24
Skin I-s	4	1	-	-	-	8	10	5	3	6	37
Skin II	2	-	-	-	2	2	17	5	2	1	31
Peritoneum I	4	-	2	-	3	1	23	3	-	1	37
Peritoneum II	5	1	2#	-	1	1	20	-	-	2	32
Uterus II	3	1	1	-	-	12	7	2	-	-	26
Uterus III	3	2	-	-	-	1	27	1	-	1	35
Right cornea	6	1	1	1	6	3	19	-	3	1	41
Left cornea	6	-	1	1	4	3	23	-	4	6	48
Right retina	7	1	-	4	4	6	12	-	-	3	37
Left retina	4	4	-	-	5	4	12	-	-	2**	31
Parathyroid (upper)	6	3	1	3	5	4	34	-	1	5	62
Thyroid	4	1	1#	-	3	2	15	-	1	3	30
Kidney	12	-	-	1	5	2	30	-	2	8	61
Left ovary	8	-	1	2	1	2	28	-	-	-	42
Right ovary	4	-	-	-	2	2	24	-	-	1	33
Spleen	5	1	-	1	2	-	18	2	-	2	31
Broad ligament	4	1	-	2	1	3	18	-	-	1	30
Intestinal mesentery	2	1	1	2	2	2	17	-	4	5	36
Lung	2	-	1	-	1	1	25	1	1	-	32
Smooth muscle (duodenum)	-	1	-	3	-	-	7	-	-	-	11
TOTAL	92	19	13	21	47	69	396	20	21	49	747
(%)	(12.3)	(2.5)	(1.7)	(2.8)	(6.3)	(9.2)	(53.0)	(2.7)	(2.8)	(6.6)	
"Broken cells" = 25.7%											
* P = polyploids. Cells were recorded only when karyotypable.											
** See text.											
# 63,XX cells.											

Table 4-7

Percentages of artifactual aneuploidy and cells with 64/28 non-acrocentric chromosome counts.

CELL CULTURE	TOTAL CELLS COUNTED	% ARTIFACTUAL ANEUPLOIDY***	% 64, XX CELLS
Skin I-ss	24	8.33	41.6*
Skin I-s	37	13.5	21.6*
Skin II	31	12.9	6.45
Peritoneum I	37	18.9	2.7
Peritoneum II	32	28.1	3.1
Uterus II	26	19.2	46.1*
Uterus III	35	14.3	2.86
Right cornea	41	36.6	7.31
Left cornea	48	25.0	6.25
Right retina	37	43.2	16.2**
Left retina	31	41.9	12.9**
Parathyroid	62	29.0	6.45
Thyroid	30	30.0	6.67
Kidney	61	29.5	8.19
Left ovary	42	28.6	4.76
Right ovary	33	18.2	6.06
Spleen	31	29.0	0
Broad ligament	30	26.7	10.0
Intestinal mesentery	36	22.2	5.55
Lung	32	12.5	3.12
Smooth muscle (duodenum)	11	36.4	0
Total		25.6	9.24

* Possible true 64,XX cell line
 ** Very high rate of tissue specific artifact
 *** Artifactual aneuploidy = percent of cells evaluated with counts of ≤ 62 , 63, and 64 (non-acrocentric 27 and 29).

cells with chromosome counts of 74, 77 and 78. An additional three cells (0.4%) which had counts of 67, 68 and 69 chromosomes were probably parts of polyploid cells. Three cells (0.4%) one each from skin II, intestinal mesentery and right cornea cultures, had a 66 (30 non-acrocentric) chromosome count. These were confirmed by chromosome morphology to be 66,XXXX cells. Twelve cells (1.6%), one each from skin I-s, skin II, left cornea, parathyroid and lung cultures; two each from right cornea and kidney cultures; and three from intestinal mesentery cultures; showed a 66/29 non-acrocentric chromosome count. These metaphases were karyotyped and by convention the 37th acrocentric chromosome was designated a Y-chromosome, and thus they were considered to be 66,XXXY cells.

Polyploid cells were only recorded when they could be karyotyped. The two polyploid cells from Left Retina cultures were 8N and 6N. Both these cells may have represented a specific factor in a particular culture. Octaploid cells can (and do) occur in-vitro through endoreduplication and/or sequential failure of cytokinesis. Cells with six haploid chromosome sets can occur, most simply, through an 'error' of division in an octaploid cell or failure of cytokinesis in a triploid cell. Although both imply mitotic instability, the 6N cell can suggest abnormal in-vivo antecedent cells (Stern, 1958). Since no other suggestion of possible triploidy was found in cultured cells from H-52, this cell was thought to be a result of an in-vitro error of segregation of an octaploid cell.

Figure IV.14 presents as an histogram the proportions of 64,XX cells (adjusted for artifact), 65,XXX and 65,XXY cells found amongst cells with chromosome counts of 64/28 non-acrocentric and 65, per tissue and over all non-lymphocyte cultured cells evaluated.

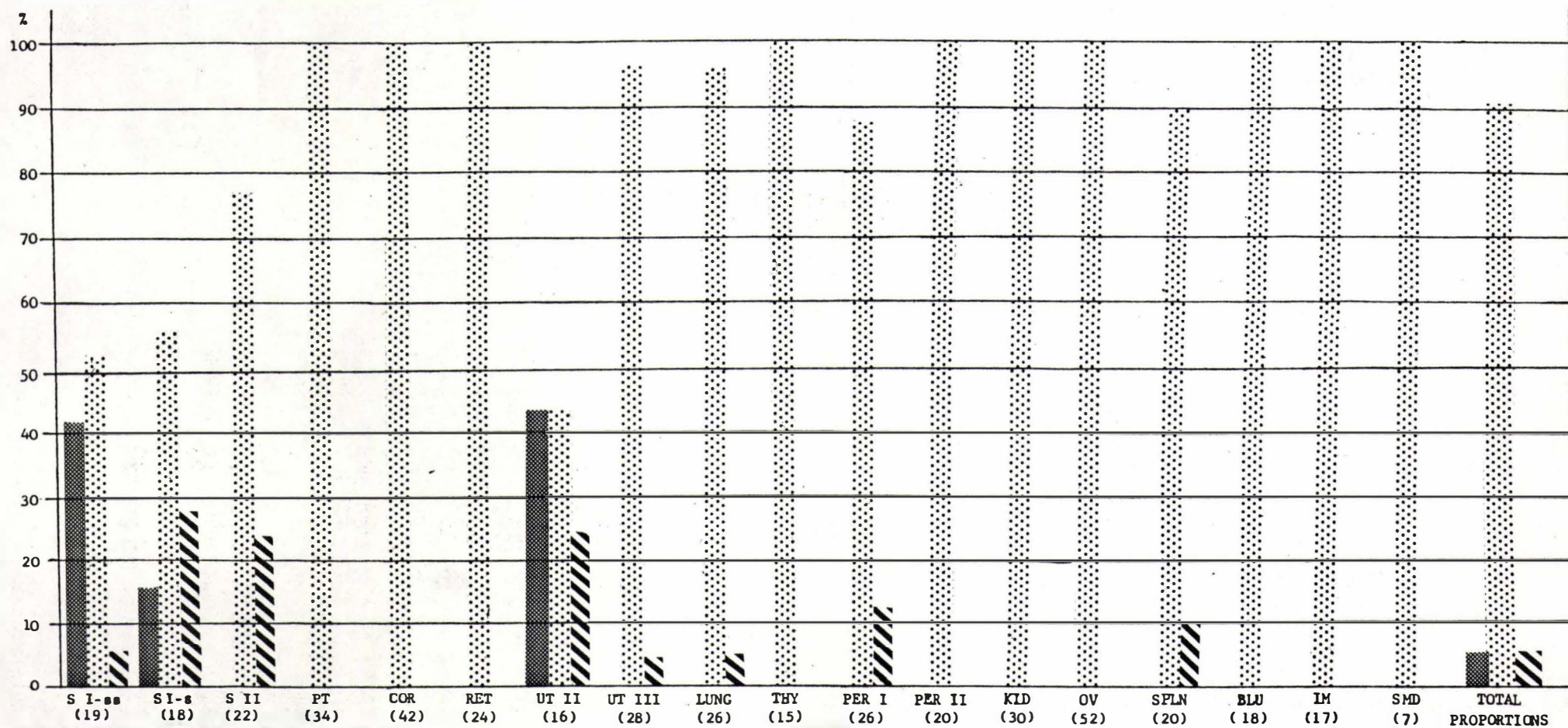


Figure IV.14

Proportions (%) of the cells evaluated from solid tissues that had, by differential count, 64,XX, 65,XXX and 65,XXY chromosome constitutions. See facing page for key to tissues.

■ = 64,XX, ● = 65,XXX and ▨ = 65,XXY.

4.3.1.3. Results of sex-chromatin studies

Sex-chromatin was examined from sections of the cervical (segments upper, middle, lower), thoracic (segments upper, middle, lower), lumbar (segments upper, lower) and sacral regions of the spinal cord, and the cerebellum. Neurons with two and three sex chromatin bodies provided direct evidence of cells containing three and four X-chromosomes respectively. Table 4-8 gives the numbers and distributions of sex-chromatin bodies. Table 4-9 repeats this information as proportions of neurons examined. Neurons with one sex-chromatin body supported the suggestion from cultured cells that H-52 had had a true 64,XX cell line. Comparison of the results from H-52 with those from a normal 64,XX mare (Blue, 1976) did not indicate that H-52 had either a 63,XO or 64,XY cell line.

Analysis of this data was performed using a R x C table for attribute data with more than one degree of freedom (Snedecor and Cochran, 1973).

No significance was found within segments of sections from cervical, thoracic and lumbar regions ($P > 0.179$, 0.585 and 0.195 respectively) for numbers of sex-chromatin bodies present. The χ^2 for the numbers of sex-chromatin bodies present for combined regions examined was significant ($P > 0.0307$, $\chi^2 = 22.809$, d.f. = 12), with the largest departure from independence contributed by the cerebellum with excess of one sex-chromatin body and cervical region with excess of cells with no sex-chromatin bodies.

The χ^2 derived from analysis of the location of the sex chromatin bodies was highly significant ($P > 0.005$, $\chi^2 = 78.49$, d.f. = 36), with the contribution of cells of the cerebellum having one sex chromatin body located peripheral to the nucleolus showing the largest share of independence and with those in the nucleoplasm the next largest share. Together these accounted for 21.7% and 20% respectively of the value. If the cerebellum was eliminated from the calculation, $\chi^2 = 53.157$, is still significant ($P > 0.011$, d.f. = 32). Twenty-five percent of this value was contributed by the sacral region with an excess of cells with one sex-chromatin body located in the nucleoplasm.

The significance of the distributions of numbers and locations of sex-chromatin bodies may have reflected differences in the mosaic/chimaeric composition of H-52's central nervous system. On

Table 4-8

The numbers and distribution of sex-chromatin bodies in neurons of H-52's central nervous system.

REGION OF NERVOUS TISSUE	NUMBER OF SEX CHROMATIN BODIES				TOTAL CELLS PER REGION	POSITIONS OF SEX CHROMATIN WITHIN THE NUCLEUS						
	0	1	2	3		One body		Two bodies			Three bodies	
						P.N.	NUC.	1-P.N.	2-P.N.	2-NUC.	1-P.N.	2-P.N.
Upper cervical	17	37	12	0	66	33	4	1	11	0	0	0
Middle cervical	23	27	20	0	70	20	7	1	18	1	0	0
Lower cervical	29	25	11	0	65	17	8	3	7	1	0	0
TOTAL CERVICAL	69	89	43	0	201	70	19	5	36	2	0	0
Upper Thoracic	15	29	21	0	65	24	5	3	18	0	0	0
Middle Thoracic	13	36	15	1	65	28	8	0	14	0	0	1
Lower Thoracic	17	33	15	0	65	24	9	2	11	2	0	0
TOTAL THORACIC	45	98	51	1	195	76	22	5	44	2	0	1
Upper Lumbar	30	53	17	0	100	42	11	2	12	3	0	0
Lower Lumbar	21	51	27	1	100	43	8	5	18	4	1	0
TOTAL LUMBAR	51	104	44	1	200	85	19	7	30	7	1	0
SACRAL	58	105	38	0	201	66	39	2	31	5	0	0
CEREBELLUM	21	64	14	1	100	64	0	1	11	2	1	0
TOTAL	244	460	190	3	897	361	99	20	151	18	2	1
(%)	(27.2)	(51.3)	(21.2)	(0.3)		(55.3)	(15.2)	(3.1)	(23.1)	(2.8)	(0.3)	(0.2)

P.N. = peripheral to nucleolus
 NUC. = in the nucleoplasm

Table 4-9

The proportions of cells with sex-chromatin bodies and their distribution in H-52's central nervous system.

REGION OF NERVOUS TISSUE	NUMBER OF SEX CHROMATIN BODIES				TOTAL CELLS PER REGION	POSITIONS OF SEX CHROMATIN WITHIN THE NUCLEUS						
	0	1	2	3		One body		Two bodies			Three bodies	
						P.N.	NUC.	1-P.N.	2-P.N.	2-NUC.	1-P.N.	2-P.N.
Cervical	0.34	0.44	0.21	0	201	0.53	0.14	0.04	0.27	0.02	0	0
Thoracic	0.23	0.50	0.26	0.005	195	0.51	0.15	0.03	0.29	0.01	0	0.01
Lumbar	0.26	0.52	0.22	0.005	200	0.57	0.13	0.05	0.20	0.05	0.01	0
Sacral	0.29	0.52	0.19	0	201	0.46	0.27	0.01	0.22	0.01	0	0
Cerebellum	0.21	0.64	0.14	0.01	100	0.81	0	0.01	0.14	0.03	0.01	0
TOTAL	0.27	0.51	0.21	0.03	897	0.55	0.15	0.03	0.23	0.03	0.003	0.002

P.N. = peripheral to nucleolus

NUC. = in the nucleoplasm

the other hand, the significance might also have arisen from regional differences in the 'behaviour' of supernumerary X-chromosomes.

Overall proportions of cells containing either one or two sex-chromatin bodies were approximately 13:24:10.

4.3.2. Reports of Examinations and Observations

4.3.2.1. Physical examinations of reproductive organs

During her stay at Massey University, periodic examinations of H-52's reproductive tract were made by Prof. D. Fielden. Over that period there were no apparent changes in H-52's reproductive organs.

There was minimal mammary gland development. The cervix was normal, centrally placed and contracted. The uterus was of normal size but had poor tone and seemed to be abnormally thin walled. An endometrial biopsy evaluated by Dr. M. Alley showed severe endometrial hypoplasia (Figure IV.15).

It was difficult to palpate the ovaries per rectum. Both ovaries were very small and were estimated to be 3 cm x 1.5 cm x 1 cm in size. Both ovaries appeared to be inactive and on no occasion could follicular structures be felt.

On 12th June, 1979, an exploratory laparotomy was performed on H-52. The uterus was exposed and examined. The ovaries could not be viewed from the laparotomy, however they were palpated while the uterus was exposed. The laparotomy findings were in agreement with those made by rectal palpation.

4.3.2.2. H-52's general medical history at Massey University

H-52 was donated to the Cytogenetics Unit for research into her chromosome and reproductive status. She arrived 1st March, 1979. Her previous medical history was not available. J. Francis, B.V.Sc., was H-52's general medical supervisor at Massey University.

On arrival H-52's condition was generally poor, although dermatophilosis lesions on the rear sock were the only specific

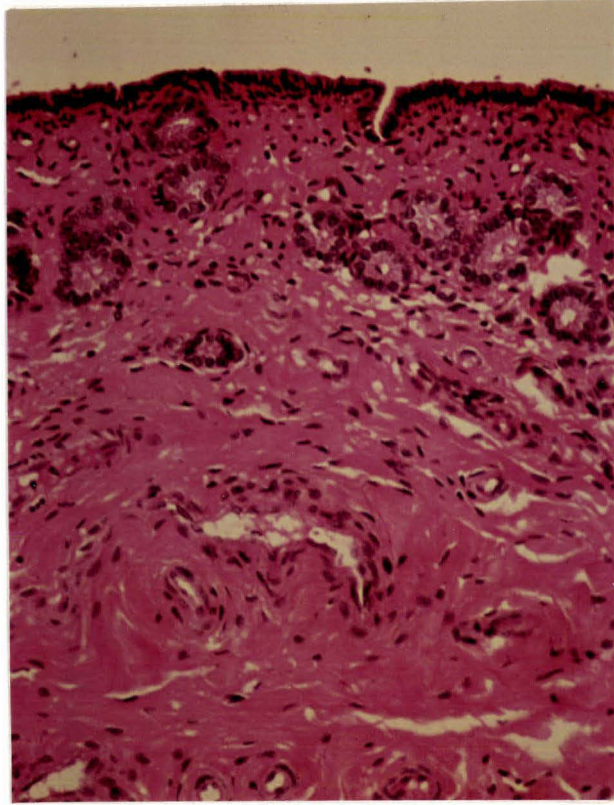


Figure IV.15

A section of uterine tissue from H-52 showing endometrial hypoplasia (X 690).

findings². These lesions were successfully treated. Subsequently, extensive dermatophilosis broke out on her flanks and back, which was also successfully treated. Late in July, 1979, H-52 developed localized sweat patches and areas of "itch" (etiology unknown) in regions that had been unaffected by the earlier dermatophilosis. This condition arose sporadically over a period of about five weeks, and while the mare was treated to relieve irritation, the condition appeared to disappear spontaneously.

The mare was drenched for internal parasites one month after her arrival.

When H-52 was destroyed, 18 September, 1979, although she had gained weight, her general condition remained poor.

4.3.2.3. Serum hormone analysis

Two serum hormone analysis trials were performed by Prof. C.H.G. Irvine, Veterinary Science Department, Lincoln College, Christchurch. (Reference Evans and Irvine, 1975, 1979.)

The first analysis, for levels of luteinising hormone (LH) and follicle stimulating hormone (FSH), was performed on serum from two blood samples taken four hours apart, at a two month interval during the normal anoestrous period. The second analysis was on a series of samples taken at the beginning of the normal breeding period. This test involved injection of 1mg GnRH (gonadotrophin releasing hormone) (P.I.M. 180, AY-24, 031 injectable, Ayerst Laboratories). Table 4-10 gives the results of these tests.

According to Prof. Irvine, the results of the LH hormone assays were consistent with those obtained from mares in dioestrus or anoestrus. Since the samples were obtained during either the normal dioestrous or anoestrous period, these results could be considered normal. One FSH level was higher than is usually found at the time of the year when sampling occurred. However, the rest were probably normal, and the mare can show great variability in FSH levels during the transitional phase between seasons. The response to exogenous GnRH, was probably a true response reflecting either a dioestrous or

²H-52's journey to Palmerston North was delayed by Industrial Action. The personnel of Majestic Horse Floats, Inc., are to be commended for the relatively good condition in which she arrived under the circumstances.

anoestrous condition and therefore disturbed function of the hypothalamus-pituitary axis was not suggested (C.H.G. Irvine, pers. comm.).

Table 4-10

Results of serum hormone analysis' I and II

<u>DATE</u> of sample	<u>TIME</u> of sample	<u>LH</u> (ng/ml)	<u>FSH</u> (ng/ml)
31.5.79	1100 hours	6.0	26.4
31.5.79	1600 hours	3.0	60.4
27.7.79	1100 hours	3.0	27.4
27.7.79	1600 hours	10.0	-
17.9.79	1405 hours*	4.0	-
17.9.79	1435 hours	14.0	-
17.9.79	1505 hours	19.0	-
17.9.79	1607 hours	11.5	-

*immediately prior to GnRH injection

4.3.3. Post-Mortem Findings

H-52 was destroyed on 18 September, 1979. She was made unconscious by an injection of barbiturates and the jugular vein was severed. The autopsy was conducted by Dr. A. Johnstone, Department of Veterinary Pathology. Details of the autopsy findings can be found in Appendix IIIB.

The mare was in poor condition. According to Dr. Johnstone there were several things wrong with the mare, however none of these individually was important enough to result in the condition seen. The majority of pathological changes found in the organs and tissues related to chronic parasitic damage. However, both the level of parasitism and the changes found were no worse than those that are found in mares, of the same breed and age, who were in good condition at the time of their deaths (A. Johnstone, pers. comm.).

Both grossly and histologically, the uterine horns, cervix and vagina appeared to be normal. The Fallopian tubes were patent. The uterus measured 16 cm from horn to horn and 12 cm from the internal os to the external vulva. This was somewhat small, but was neither an unusual nor an abnormal size for a mare which never had had a foal and was neither pregnant nor in oestrus at the time of death. With the exception of mild chronic focal endometritis, with associated glandular atrophy, the tissues of the uterine body were essentially normal. In view of the inspissated contents of the glands of the uterus, the histological changes that were seen were thought (according to Kenney's 1978 work) more likely to represent either anoestral or degenerative atrophy rather than congenital hypoplasia (A. Johnstone, pers. comm.).

Photographs of H-52's genital organs are presented in Figures IV.16, IV.17 and IV.18.

The left ovary (Figure IV.19) measured 4 x 2 x 2.5 cm. It contained a round area of orange luteal tissue, with a central brown area, 2 mm in diameter. One or two brown haemorrhagic areas were present. Histologically there was no evidence of follicles. However, there was an area of local haemorrhage, between 14 days and several months old (corresponding to the brown haemorrhagic areas seen macroscopically), which was both Pearl's Iron and Sudan Black positive (Figure IV.20). These positive stain reactions supported the belief that this was a corpus albicans.

The right ovary (Figure IV.21) measured 3.5 x 2.5 x 1.5 cm. A paraovarian cyst was adjacent to it in the mesovarium. Brown haemorrhagic spots were visible macroscopically. Histologically these were areas of scarring with a centrally large area of haemosiderin and some cells filled with light brown granular material that were both Pearl's Iron and Sudan Black positive (Figure IV.22) and thus indicated that they were Corpora albicantia. There was no evidence of follicles and only stromal tissue was present.

The tissue layers were normally organized. In both ovaries there were isolated Sudan Black positive cells, indicating that there was some ovarian function. The haemosiderin indicated an area of old haemorrhage and the scarring found probably represented the organized area in the middle of the corpus haemorrhagicum, while the presence of Sudan Black positive cells suggested probable luteal cells. Therefore, although no follicles were seen in the histological sections, the mare probably had ovulated (more than once) within the last several months (A. Johnstone, pers. comm.).



(left) *Cornu uteri*

(left) *Ovari*

Ligamentum latum uteri

Broad Ligament of Uterus

Corpus uteri

(uterine body)

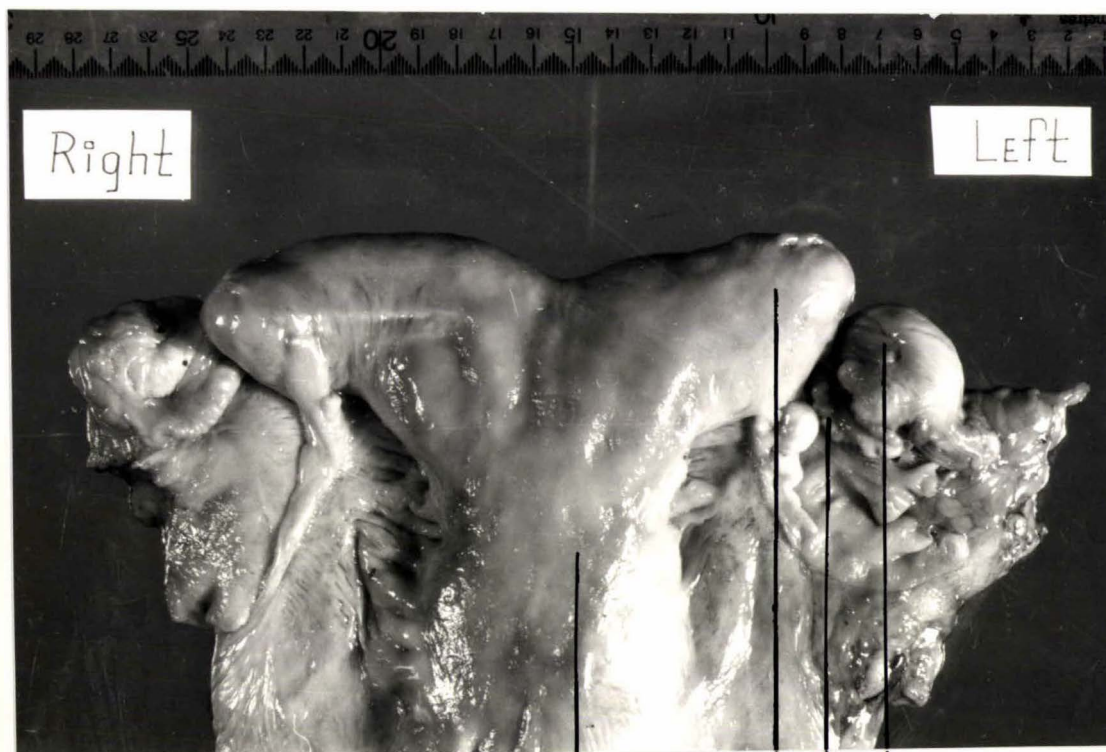
urinary bladder

Urethra feminina

(urethra)

vagina

Labia vulvae of the Rima vulvae
(vulva or vulvar cleft)



- Ovari
(ovary)
- Tubae uterinae
(Fallopian tube)
- Cornu uteri
(uterine horn)
- Corpus uteri
(body of the uterus)

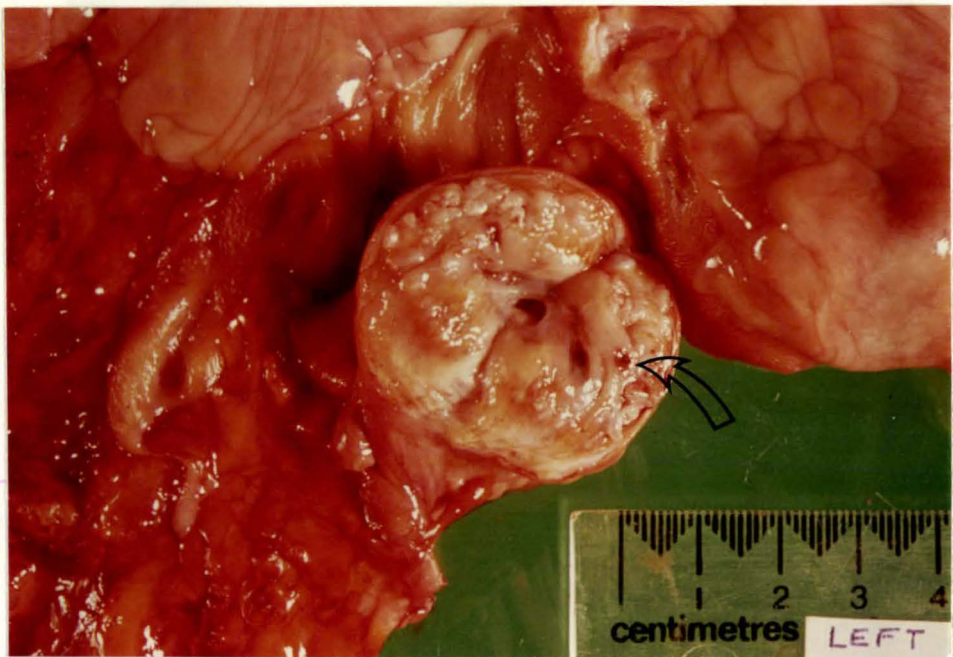
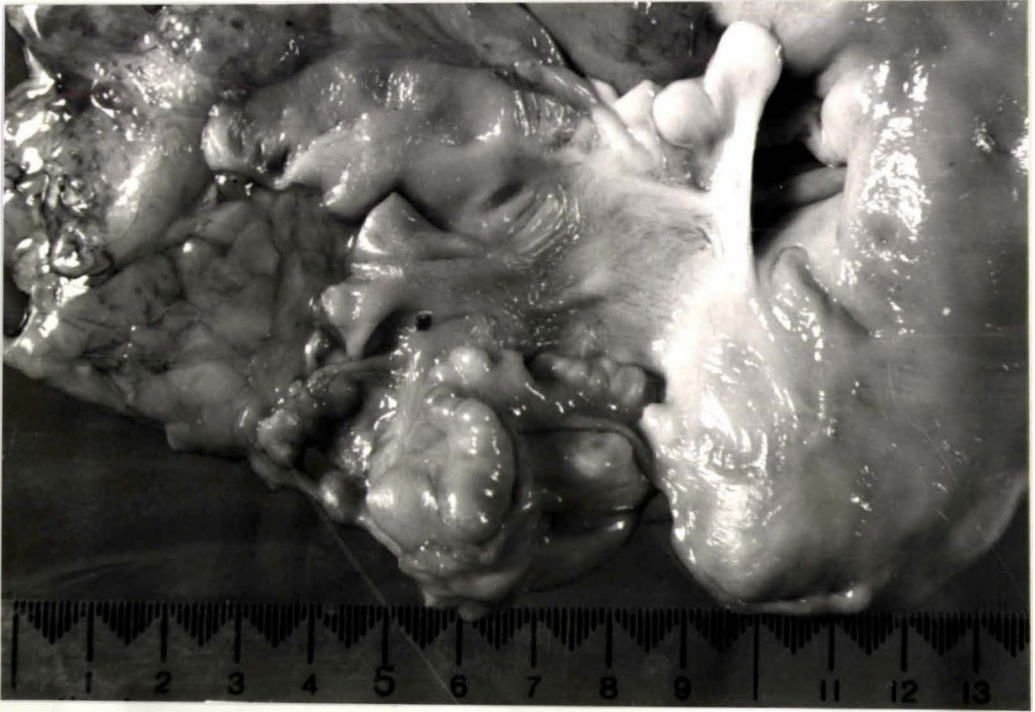


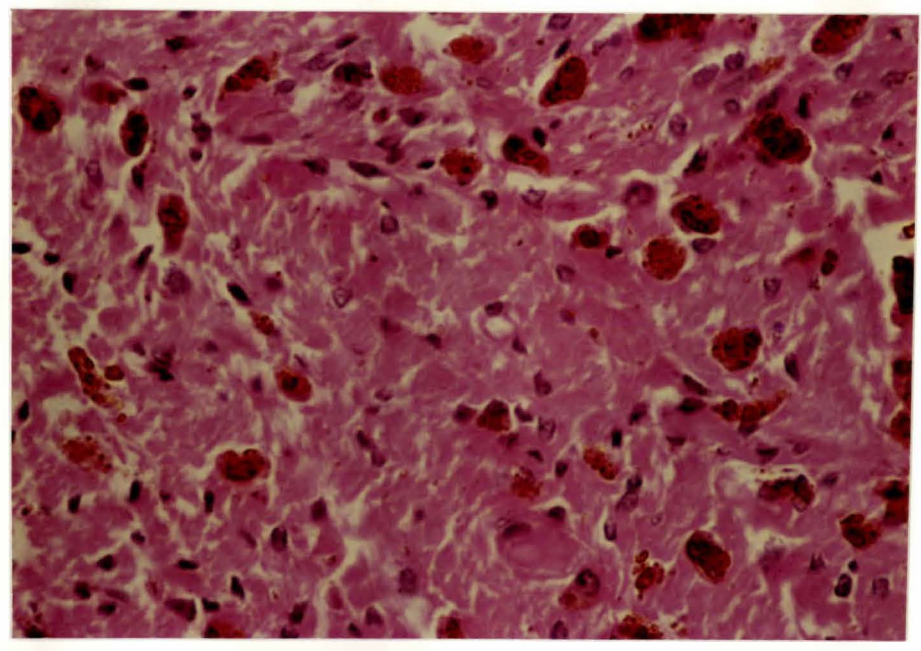
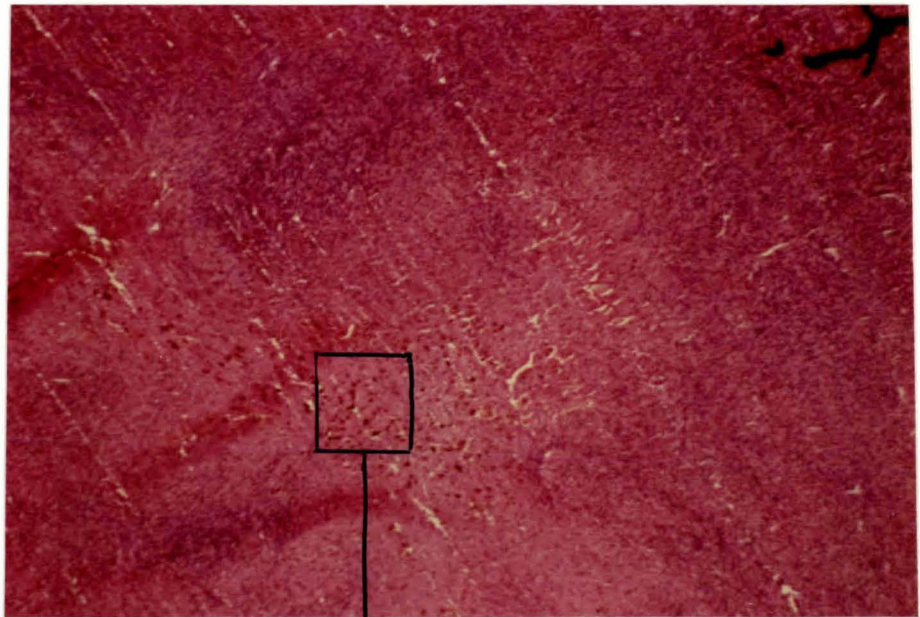
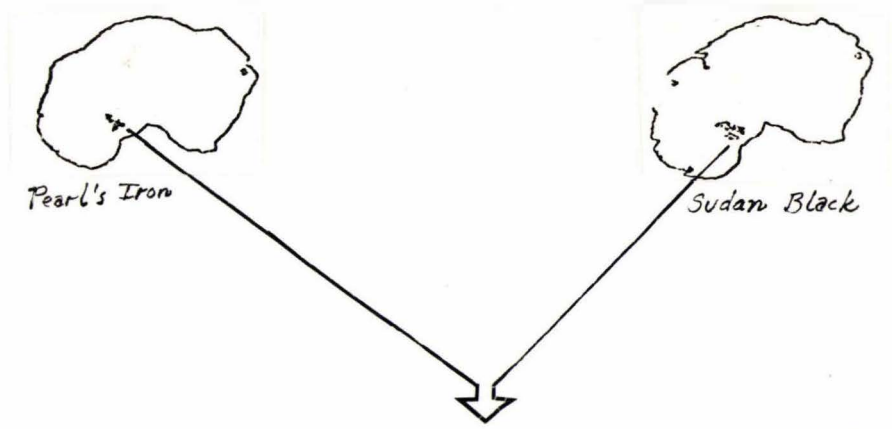
Cornua uteri
(uterine horns)

Corpus uteri
(uterine body)

Cervix uteri
(cervix)

vagina





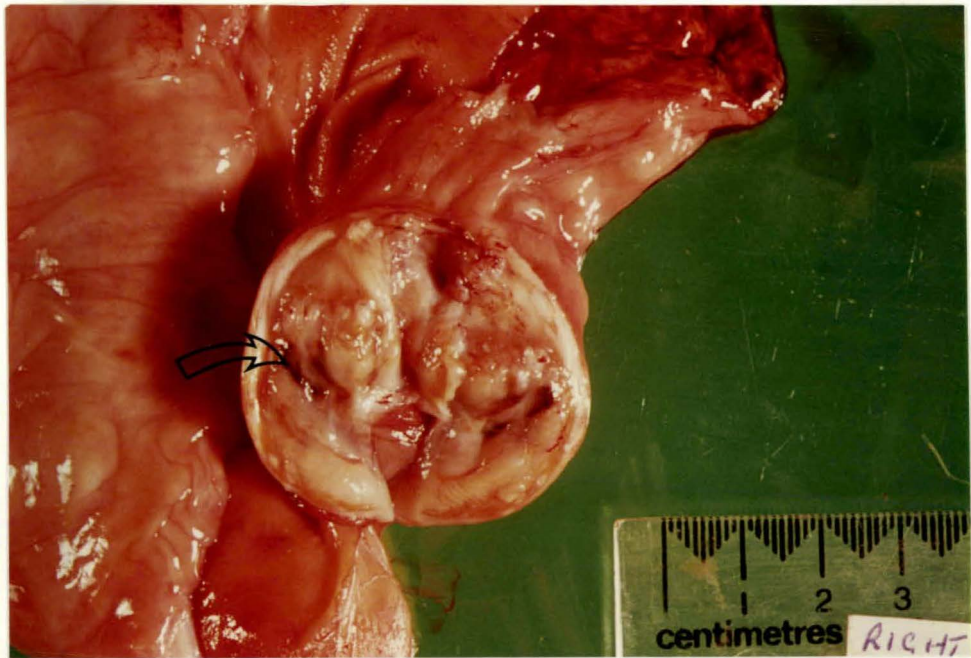
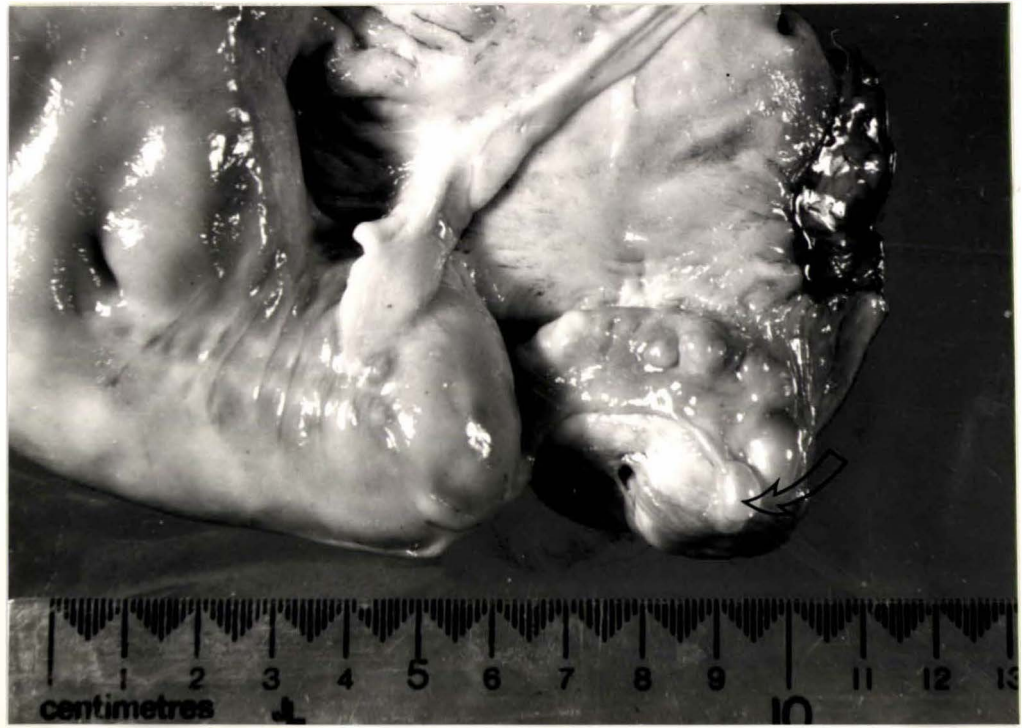
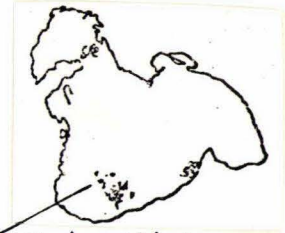


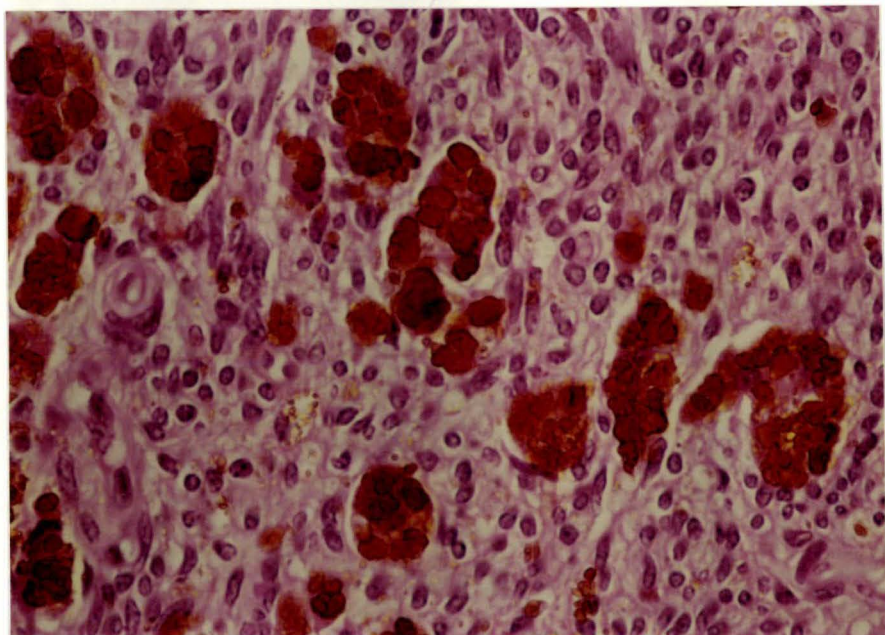
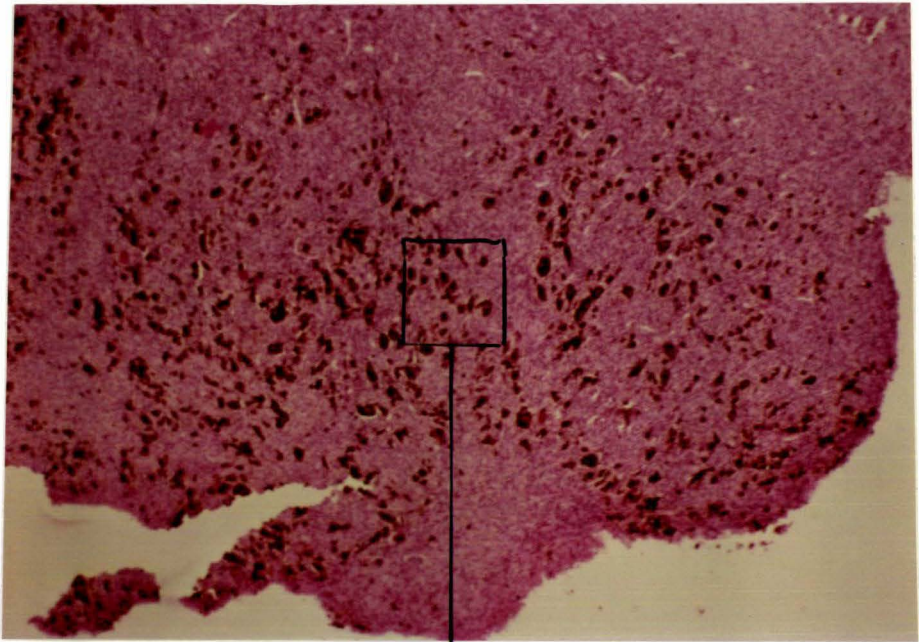
Figure IV.22 Drawings of Pearl's Iron and Sudan Black positive areas of right ovary sections (X2), and two photographs of a region of one of these areas (X 160 and X 1,400) in eosin-haematoxylin stained sections. These were believed to be areas of corpus albicans s. fibrosum.



Pearl's Iron



Sudan Black



4.3.4. Observations on H-52's Behaviour

H-52 generally moved in a sluggish manner, with her head down and dragging her feet. She was never observed to run, although on occasion she did prance or could be encouraged to walk quickly. Following testing by Dr. H. Pearce, it was suggested that H-52 might be showing a mild ataxia. She was not sure footed, however, this may have been due at least in part to the poor condition of her hoofs. H-52 tended to bump into objects or step on them. She seldom reared, though on more than one occasion when she did, she lost her balance and fell.

When not grazing or when boxed, H-52 would almost invariably stand with her right side against the fence or wall. Pawing motions were usually made with the left forefoot. She kicked most often with the left rear foot, in a lateral direction (even when the right would have been more convenient and effective).

She tended to bolt her feed and she was noted to slobber and dribble food while chewing. In the fields and paddock H-52 would graze the area within reach of her mouth before moving, and then it was rare for her to move more than ten centimeters. In the paddock H-52 did not have regular sites for defaecation.

H-52 often displayed "play" behavior as defined by Waring (1975). She also tended to bite people and objects, and went through episodes of 'crib biting' her paddock fence.

Despite her physical condition, H-52 always appeared to be alert and persons having direct contact with her considered her to be a cunning mare. H-52 appeared to identify certain objects such as the Massey tractor, vacutainers and a particular plastic squeeze bottle. Her recognition of the author seemed to be independent of any particular garment that was worn. According to Dr. R. Holmes, H-52 was hypersensitive to some stimuli (or had a low threshold for stimulation) and was hyper-reactive to others.

4.4. Discussion

4.4.1. Cytogenetic Aspects of H-52's Mixoploidy

From the wide variety of tissues examined, including the sex-chromatin studies, it was concluded that H-52 was a 65,XXX/65,XXY/64,XX/66,XXXY/66,XXXX mixoploid. There are several ways the mixoploid cell lines found in H-52 might have originated. It is axiomatic that the most probable way is the one requiring the fewest steps or errors, each of which has the highest probability of occurring. Extrapolation from an estimate of the chromosome constitution of a mature animal to its zygotic or embryonic chromosome constitution is fraught with assumptions. Foremost of these assumptions are: that the condition of the mature animal, as sampled, reflects that of the animal; and that the condition of the mature animal reflects that of the zygote or embryo.

4.4.1.1. The chromosome constitution of H-52

It was not possible to locate in the literature either similar or comparable mixoploids where the behavior of supernumerary X-chromosomes in neurons had been studied. Therefore, sex chromatin studies on H-52 were considered only supportive and confirmatory of her chromosome constitution as ascertained using cultured cells.

Overall, 21% of the neurons examined from H-52 had two sex-chromatin bodies. The presence of two sex-chromatin bodies indicates two inactive X-chromosomes and therefore was direct evidence of cells with three X-chromosomes (either XXX or XXXY). Three sex-chromatin bodies were present in 0.3% of the neurons examined, confirming the presence of cells containing four X-chromosomes.

H-52 showed an overall incidence of 72.8% of neurons with one or more sex-chromatin bodies. This compared with an overall incidence of 70% of neurons with one sex-chromatin body in a normal 64,XX mare when the same technique was used (Blue, 1976; Blue *et al.*, 1978). There was no suggestion, therefore, that H-52 was mosaic/chimeric for either a 63,XO or 64,XY line in her neuronal cells. There had been no suggestion of either of these lines in chromosome preparations from H-52's cultured cells.

Fifty-one point three percent of neuronal cells evaluated for sex-chromatin from H-52 had one Barr body, 40.2% of which were adjacent to the nucleolus. The presence of one Barr body indicates the presence of one inactive X-chromosome, but does not differentiate between XX and XXY cells.

Without control sex chromatin studies of neurons from mixoploids similar to H-52, the relationship between the results from the sex-chromatin investigations and the mosaic/chimaerism actually present in her nervous tissue could not be determined. Four points, however, suggested that either the mixoploid combination as evidenced by cultured cells was greatly different from that of nervous tissue as evidenced by the sex-chromatin studies, or that as a gauge of mosaic/chimaerism of H-52's type, neuronal sex-chromatin investigations are insufficiently refined. These points were: (1) the scarcity of XX cells and their possible limitation to epidermal and/or dermal and lymphatic tissues (with the exception of one uterus culture where there was a strong possibility of culture effect); (2) the relatively few 65,XXY cultured cells and their wider intra-tissue distribution than 64,XX cells; (3) the scattered occurrence of the twelve 66,XXXY cells; and (4) the three 66,XXXX cultured cells, an incidence in adjusted non-lymphocyte cultured cells of 0.33%, against an incidence of 0.4% of neurons containing three Barr bodies.

Therefore, it is assumed (for the remainder of section 4.4.1) that the results from cultured cells provided an accurate estimate of H-52's mosaic/chimaeric composition. That is, that the XXX line was overtly predominant to all others, that the other cell types occurred in H-52 in approximately the same relative proportions as were found in in-vitro cultured cells, and that she had neither a 64,XY nor 63,XO cell line.

4.4.1.2. The possible zygote/embryonic chromosome constitution of H-52

The presence of cells containing a Y-chromosome in tissues not of the reticulo-endothelial system excluded the possibility of their post-zygotic addition to the embryo. Therefore, the Y-chromosome must have been contributed at the conception of the H-52 zygote (if mosaic) or zygotes (if chimaeric). Although H-52's dam was not available for cytogenetic analysis, her reproductive record was normal. So, until evidence is available on the existence and

functional ability of "Y" containing oocytes in mares, it must be assumed H-52 received her Y-chromosome from her sire³.

According to the theory that there is a proliferative advantage inversely proportional to the amount of heterochromatic sex chromosome material present (Barlow, 1972, 1973; Mittwoch and Delhanty, 1972), the predominance of 65,XXX cells and scarcity of 65,XXY and 64,XX cells in H-52 implied that at (at least) the time of X-inactivation the XXX blastomeres greatly outnumbered either XXY or XX ones. The asynchronous cleavage that occurs in Equidae (McLaren, 1974) is believed by embryologists to be determined solely by the amount and location of the yolk in the ovum. If this was so, any quantitative differences in the types of blastomeres that formed H-52's embryonic mass would not have been due to properties intrinsic to their chromosome constitutions. For random segregation to have resulted in an embryonic mass containing a predominance of XXX blastomeres, suggested that the pre-blastocyst H-52 was mainly of triple-X chromosome constitution relative to the XXY or XX components.

Possible support for the supposition that the chromosome configuration of the majority of H-52's blastomeres was triple-X came from estimated primordial (precursor) cell pool sizes. Primordial cell pool sizes estimated by analysis of XXX, XXY and XX variation in the mosaic/chimaeric composition of H-52's cultured cells suggested that the 65,XXX line arose first followed by the 64,XX and 65,XXY cell lines (see Appendix IIIC).

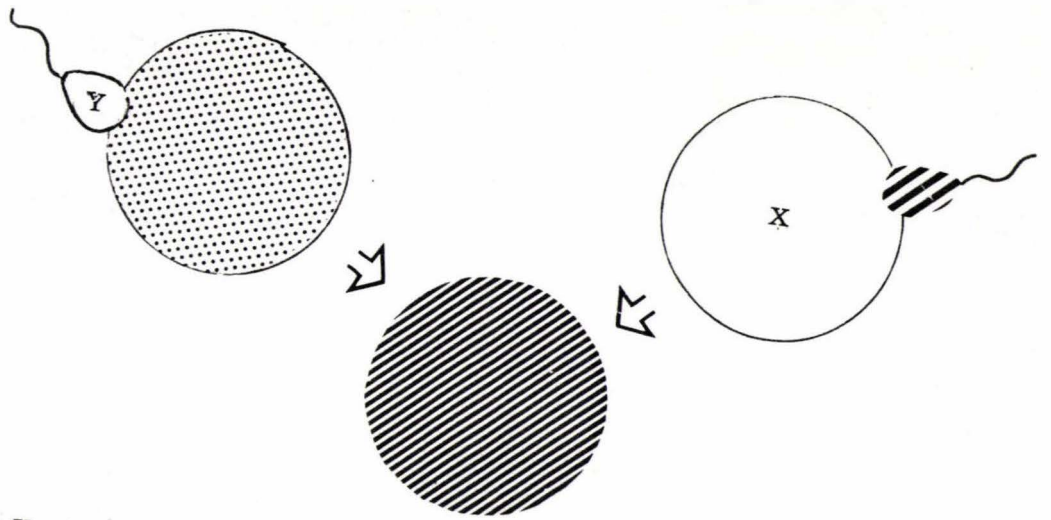
There are two possible ways that both the XXX line could have been predominant and the XXY line could have arisen later than the XXX line: (1) The XXX line arose at the 1st or 2nd cleavage division, and the XXY line at a later one, in a conceptus whose chromosome configuration was different from either (Mosaic manner); and (2) The XXX and XXY blastomeres were from two conceptual events, at different stages of blastogenesis, that fused (Chimaeric manner).

³ Even if Sharp et al.'s (1980) XY mare, who was reported to have foaled a normal 64,XX filly, proved to be an example of functional XY oocytes in a mare, proof would still be required that random meiotic segregation could occur in such ova.

The Chimaeric manner of formation was the least probable. Both non-disjunction at meiosis I of a normal XX ovum with dispermic double fertilization of the egg nucleus and a polar body (on the order of Zuelzer et al., 1964) and fusion of two separate conceptuses at different cleavage stages, could provide possible explanations for the predominance of the XXX line and the presence of the XXY cell line. However, there is an extremely low probability that two separate abnormal conceptuses would both form and fuse. Although it is within the realm of probability that an XX and an XY conceptus could fuse, the series of mitotic errors necessary to account for all the cell lines present in H-52 (and possibly the absence of XO and XY cell lines) from a XX/XY chimaera make this origin improbable. On the other hand, in the absence of genetic proof that H-52 was a chimaera, dispermic double fertilization giving rise to a XXX/XXY chimaera, with subsequent mal-segregation and loss of an XY cell line, must remain a possibility.

The most probable manner of the formation of H-52's mixoploidy was mosaic. This was also the most simple mechanism to account for all the lines present in H-52, as is presented in Figure IV.23. That is, formation of a 66,XXXXY zygote, with first cleavage loss of a Y-chromosome resulting in XXX/XXXXY mosaicism and subsequent development of XXY, XX and XXXX lines.

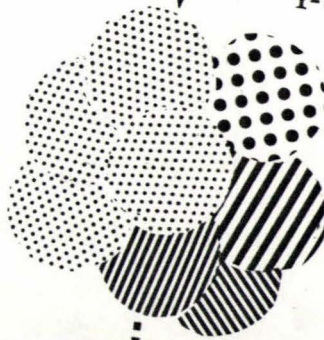
Had the events giving rise to H-52's mosaic/chimaerism included the presence or formation of 64,XY and possibly 63,XO blastomeres (which are known to be highly viable in the non-mosaic mare), one could expect some evidence of cell lines derived from these in the adult. It was possible that 64,XY and/or 63,XO blastomeres were formed and either segregated exclusively to extra-embryonic membranes or cells derived from them were limited to tissue primordia not sampled during cytogenetic investigation of the adult. It was also conceivable that 64,XY and 63,XO blastomeres could have been inviable as primordial cells. H-52's estimated primordial cell pool sizes suggested that her mixoploidy arose long before X-inactivation, as is thought to be the case in human sex chromosome mosaics (Nesbitt and Gartler, 1971; Gartler and Andina, 1976). However, even if a mechanism such as preferential X-inactivation (e.g. Monk, 1978) was involved in the loss of (functionally YO or null-0) XY or XO cells, there would have had to have been inexplicably few XY or XO blasto-



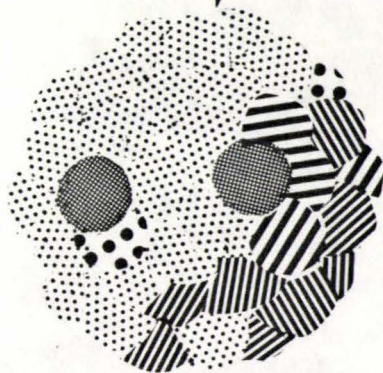
Y-chromosome loss



non-disjunction and loss of Y-chromosome in xxx blastomere



non-disjunction in xxx blastomere



Y-chromosome loss from xxy blastomere

meres. Thus, these alternatives also implied that relatively few XO or XY blastomeres could have existed, and if they did they were formed relatively late during blastogenesis.

On the other hand, perhaps in a complex mixoploid such as H-52, only those cells in differentiation phase (including compatible cell cycles) which comprise a critical majority of cells, can participate in crucial stages of tissue specific development. Assuming the survival and proliferative advantage of 64,XX cells, their scarcity in H-52 would be in agreement with such a hypothesis.

4.4.1.3. Possible predisposing factors to H-52's mixoploidy

No suggestion of factors which might have predisposed to the formation of H-52's mixoploidy was evident in her history. Maternal age effect has been suggested as involved in the etiology of a majority of individuals with multiple X-chromosomes (e.g., Hamerton, 1971, Emery, 1977) and an hereditary tendency to non-disjunction was implicated in one cattle triplo-X and related XXY bull (Reick *et al.*, 1970).

At conception H-52's sire and dam, aged nine and seven respectively, were in their reproductive prime. As far as could be ascertained, the sire's reproductive record was good. The dam's history was normal. H-52 had two older full brothers and was the dam's third consecutive foal. The year following H-52's birth the dam did not become pregnant, but in the next two years she produced (to another sire) two foals, a colt and one of unrecorded sex which died as a foal.

H-52's coefficient of inbreeding, calculated on a pedigree of over four generations, was low for a comparably well bred Thoroughbred. While she was distantly related to some animals which figure in pedigrees of other chromosomally abnormal animals that have been investigated, there was no evidence of an hereditary tendency for non-disjunction in her family.

4.4.2. Physical and Behavioural Aspects of H-52

The sole triple-X mare in the literature was a five year old Thoroughbred reported by Chandley *et al.* (1975b). This animal displayed irregular oestrus and produced, on one occasion, a single follicle. Her uterus was small and immature with an hypoplastic

endometrium. Her ovaries were small, less than 2 cm in diameter, and a biopsy of one showed only stromal tissue.

Two triple-X cattle have been reported. One, a Norwegian Red Cattle Breed heifer, assumed to be infertile, was reported by Norberge et al. (1976). She was somewhat smaller than normal size with atrophic, underdeveloped endometrium, and small underdeveloped ovaries. At 13 months of age this animal showed one heat and at slaughter, five months later, a persistent corpus luteum was found on the right ovary together with one secondary follicle. No follicles or structures were found on the left ovary. The second triple-X was a Fleckvie heifer, studied by Reick et al. (1970, 1974). This heifer was of normal size with kyphosis, a hereditary congenital defect. She was maternal aunt to an XXY bull calf and dam to a normal 60,XY calf. In both bovine cases multiple leucocyte cultures and cultured skin cells were examined to limit the probability of mosaicism.

In women, no consistent phenotype is evident in the, so called Triplo-X syndrome. The majority of 47,XXX women are thought to be fertile, though some may be at high risk of conceptual loss and the ovaries of some have shown precocious follicular depletion (Johnson et al., 1961; Barr et al., 1969; Tennes et al., 1975; Dewhurst, 1978). Three often reported features in triple-X women are: somewhat below normal motor coordination; a general slowing of all functions; and a risk of sub-normal intelligence and/or moderate to severe psychological problems (Johnson et al., 1961; Barr et al., 1969; Tennes et al., 1975).

In comparison physically H-52 was of normal size and conformation for a Thoroughbred mare. Apart from an absence of follicles and endometrial hypoplasia, no significant physical anomalies were discovered in H-52. Her ovaries appeared to be inactive, though she was reported to have cycled once and a follicle was said to have been palpated. Post-mortem investigations confirmed that some follicular activity existed and a presumptive luteinized follicle (Figure IV.19b) was observed at autopsy, which unfortunately eluded isolation for histological inspection. H-52 had extreme endometrial hypoplasia and a somewhat small, immature, uterus.

While H-52 displayed some ovarian function, her extreme endometrial hypoplasia did not suggest that she had the ability to carry a foal (Kenney, 1978). In comparison to Barone (1979) and to an anoestrous mare with a somewhat small but essentially normal

uterus (H-27, Chapter VI), H-52's uterus would seem to have been distinguished by a significant decrease in the transverse furrows which normally arise at the end of the first year. One might speculate as to whether a localized developmental arrest, perhaps related to hormone imbalance, occurred about that time. It was interesting that ovarian involution, which occurs during the same period and may be in part under the influence of some of the same hormones, did occur normally in H-52.

An increased level of FSH (follicle stimulating hormone) and LH (lutening hormone) are associated with both immature and aged ovaries, as well as with the XO condition in mares (Evans and Irvine, 1979). However, there was no evidence from the hormone assays either for or against abnormal levels of FSH and LH being involved in H-52's infertility. There was the suggestion, however, that the hypothalamus-pituitary-ovarian hormone interaction might have functioned within normal limits. Mosaic, or presumptive mosaic, women who have functioning ovaries are generally characterized by irregular menstrual cycles. H-52's history did not suggest that she displayed normal oestrus.

Human beings are the only species where non-morphological aspects of individuals with chromosome anomalies have been studied extensively. Problems occur when attempting to relate observations about H-52 with reports of triple-X women. Most objective definitions of an abnormality in humans require either neuro-physiological knowledge not yet available for the horse or intellectual and manipulative skills that are biologically impossible for non-humans. However, it was thought that comparisons should be attempted, since it was believed that any suggestion of a similar basic manifestation of an abnormal karyotype, whether the manifestation itself was 'normal' or 'abnormal' for a particular species, could be important in eventually tracing the mechanism of the dysfunction.

In the absence of objective evidence, or any subjective suggestions to the contrary, it was thought that H-52's sight, hearing and olfactory senses were normal. H-52 appeared to be an alert animal of, at least, normal intelligence. Although triple-X women have been suggested to be at high risk of being of subnormal intelligence or mentally retarded, these features are not thought to be characteristic of the XXX condition in humans (Tennes et al., 1975).

H-52 seemed to exhibit slight motor ataxia, and in general her manner of movement (i.e., slowness, clumsiness) were said to be unusual for a Thoroughbred mare. No medical reasons could be found for this aside from her general poor condition and the probable partial contribution of the poor condition of her hoofs. On the other hand, neither while she was under regular medical examination and supervision nor at autopsy, was an explanation found for either the lack of improvement in her condition while at Massey University or sufficient reasons for her poor condition. In this regard Tennes et al.'s (1975) report of poor gross motor coordination and muscle tone in XXX infant and toddler girls might have been relevant, particularly since their criteria of diagnosis closely approached that used by those appraising H-52's movements.

H-52 was observed to display the possibly atypical, but not necessarily abnormal, equine behaviours of not having specific sites in her paddock for defaecation and of exhibiting relatively immobile grazing patterns. Her playfulness and perhaps her method of eating, although possibly not abnormal in mature horses nor specifically 'foal behaviour', was interesting in view of the occasional reports of 'immature' behaviour in triple-X girls and women (e.g., Barr et al., 1969; Tennes et al., 1975; Ratcliffe, 1976).

It was unfortunate that neuro-psychiatric equivalencies did not exist between human and horse. The subjective reactions of persons who dealt with H-52 suggested that she possessed unusual responses and behaviour for a mare. Specifically, H-52's apparent hypersensitivity and hyper-reactiveness (as well as perhaps a neurological component of ataxia) could have been observable manifestations of the atypical neurologic development and/or function which Polani (1967) proposed was concomitant to cytogenetic abnormalities in humans.

H-52 was not a simple triple-X individual. It was thought, because of the predominance of the XXX line and H-52's normal female anatomy, any influence by the minor lines had probably been limited either to early embryogenesis or discrete cellular regions. No suggestion of masculinity was observed in H-52, except a tendency to bite which is more common in colts and stallions than in mares. It was not possible to locate a report of an XXX/XXY/XX/XXX/XXXX mixoploid of any species for comparative purposes. The only reports that were available on XXY mosaic/chimaeric females, with no signs of intersexuality, are presented in this Thesis (Chapter V). Therefore,

the question of what direct effects H-52's other lines may have had on her fertility could not be answered.

4.4.3. Summary

It was considered most probable that H-52 was primarily an XXX mare with XXY/XX/XXXY/XXXX mosaic mixoploidy, which arose from a 66,XXX Y conceptus. If there was any influence from her Y-chromosome bearing cells, it was thought that it was limited to indirectly mediating responses (for example inhibition of hormonal stimulation) in the direction of infertility.

The nature of H-52's reproductive deficits did not appear to be congenital. The ovaries had involuted normally and there was some ovarian activity. Similarly, the condition of her uterus might have been due either to a post-natal failure of development or degenerative changes. H-52's entire physical picture might have raised questions of possible premature senescence.

There was evidence suggesting that H-52 had produced ova, and it was reported that she had (at least once) been in oestrus, but it was not known if she was ever mated. Examinations of H-52 as a seven year old indicated that she was then probably infertile. However, early reproductive senescence may not be unusual in women with sex chromosome abnormalities, so on the basis of the limited evidence available it was prudent not to attempt estimating her reproductive ability when younger. It could be conservatively stated that H-52 would never have been more than sub-fertile.

Some aspects of H-52's phenotype (e.g., behaviour) were suggestive of atypical development, that might have related either to her specific chromosomal constitutions or to her having an abnormal chromosome constitution.

CHAPTER V

TWO 64,XX/65,XXY MOSAIC/CHIMAERIC MARES

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CHAPTER V

TWO 64,XX/65,XXY MOSAIC/CHIMAERIC MARES5.1. Introduction

The two 64,XX/65,XXY mosaic/chimaeric mares, H-46 and H-47, presented in this chapter were diagnosed on the basis of chromosome preparations from cultured lymphocytes and cell cultures initiated from skin biopsies. They have been combined into one chapter because of their similar levels of XXY mosaic/chimaerism and the limited cytogenetic (and general) information obtained about these mares. Both mares were valuable Thoroughbreds, and in addition one mare resided a considerable distance from the laboratory, so access to them for cytogenetic investigation was restricted.

In the XX/XXY mosaic/chimaeric individuals in the literature, some degree of intersexuality had always been found (e.g., Ferguson-Smith, 1966, Dain and Bridges, 1978; Dain and Walker, 1979). Neither H-46 nor H-47 presented physical signs of intersexuality. H-46 was infertile but there was insufficient information to assess H-47's breeding potential. Both mares, however, might have fallen within the category of "shy breeder".

H-47 had had two signs of defective caudal axis development, atresia ani and rectovaginal fistula. In addition the possibility was raised that H-47's high set (possibly short) tail and deformed "rump" might have indicated skeletal anomalies. H-46 had no ascertainable defects.

5.2. Materials and Methods

5.2.1. Sources of Information

Information about H-46 and H-47 was supplied by their owners and veterinarians who had attended them. Personal observations by the author were also recorded. Anecdotal information was either verified in official records or (unless otherwise noted) independently supplied by at least two reliable persons.

5.2.2. H-46

5.2.2.1. Description

Sex:	female
Foaled:	1969
Breed:	Thoroughbred
Colour:	Brown
Size:	158.125 cm (15.3½ h.h.) at withers
Age of sire at birth:	5 years
Age of dam at birth:	11 years

H-46 is shown in Figure V.1.

5.2.2.2. Feminine structures

H-46's ovaries, by palpation per rectum, were of normal size (left ovary 6cm x 4cm; right ovary 7cm x 4cm). They were reported to have usually felt like ovaries of a normal anoestrous mare. Occasionally, a small (10-15mm) follicle was said to have been felt on one or both of her ovaries.

H-46's cervix was rather flat but centrally placed and it did not protrude. There was slight relaxation of the cervical opening. There was some sinking of the dorsal commissure of the vulva. The uterus had no tone, and an endometrial biopsy showed, "severe endometrial hypoplasia similar to that seen in XO mares"¹.

5.2.2.3. Reproductive history

H-46 was not known to have ever conceived. However, there were discrepancies in reports about attempts that had been made to get

¹Dr. M. Alley.



Figure V.1

H-46 as a nine year old.

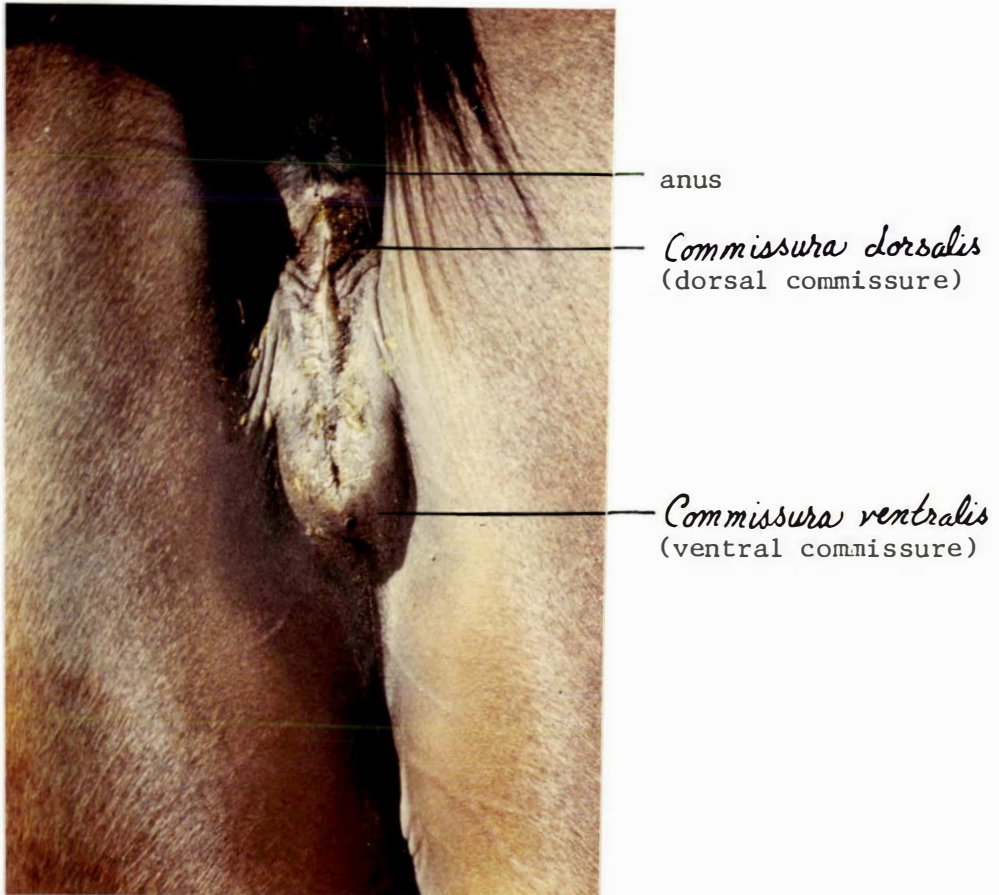


Figure V.2 Vulvar cleft (*Rima vulvae*) and anus of H-46.

her in foal. It was reported that serious attempts to breed with her had been made for two seasons (when she was aged seven and eight) but with no success. Track records showed that H-46 was racing during one of those breeding seasons, which probably would have adversely influenced attempts to breed with her. It was also reliably reported that H-46 had once been to stud for one month (when she was seven), where she was not served because she was indifferent to teasing and was never in true oestrus.

5.2.2.4. Behaviour

H-46's owner maintained that she had, at times, displayed true oestrus and according to one of her trainers H-46 seemed to be continuously in season when in racing trim. Veterinarians who have attended her were unable to confirm that, within a five year period, she had showed obvious signs of oestrus.

It was reported by her owner and trainer that when in training she displayed stallion-like behaviour such as arching of the neck. The owner stated that track commentators and officials had tended to refer to H-46 as "he" or "him".

H-46 was easily handled and was generally placid.

5.2.3. H-47

5.2.3.1. Description

Sex:	female
Foaled:	1976
Breed:	Thoroughbred
Colour:	chestnut
Size:	157.5 cm (15.3 h.h.) at withers as a three year old
Age of sire at birth:	5 years
Age of dam at birth:	8 years

H-47 is shown in Figure V.3.

5.2.3.2. General history

At two years old H-47 was small when compared with other mares of the same age on the property. It was reported that this difference in size had existed since she was a few weeks old. She was said to have poor Thoroughbred conformation and was described as



Figure V.3

H-47 as a two year old maiden mare.

disproportionate. H-47's sacral (rump) region appeared to be malformed (Figure V.4a). Her tail appeared to be set higher on her back than usual and the tail itself to be 7.5 cm shorter than is usually seen. Two hard enlargements on the left side of her neck (approximately 12.5 cm in diameter) were evident when she was two years old. Her veterinarian suggested these might represent malformations of neck ligaments. When she was three, her veterinarian reported that the vertebrae of the neck "exhibited evidence of abnormalities". It was not possible to confirm or establish the exact nature of these possible skeletal anomalies.

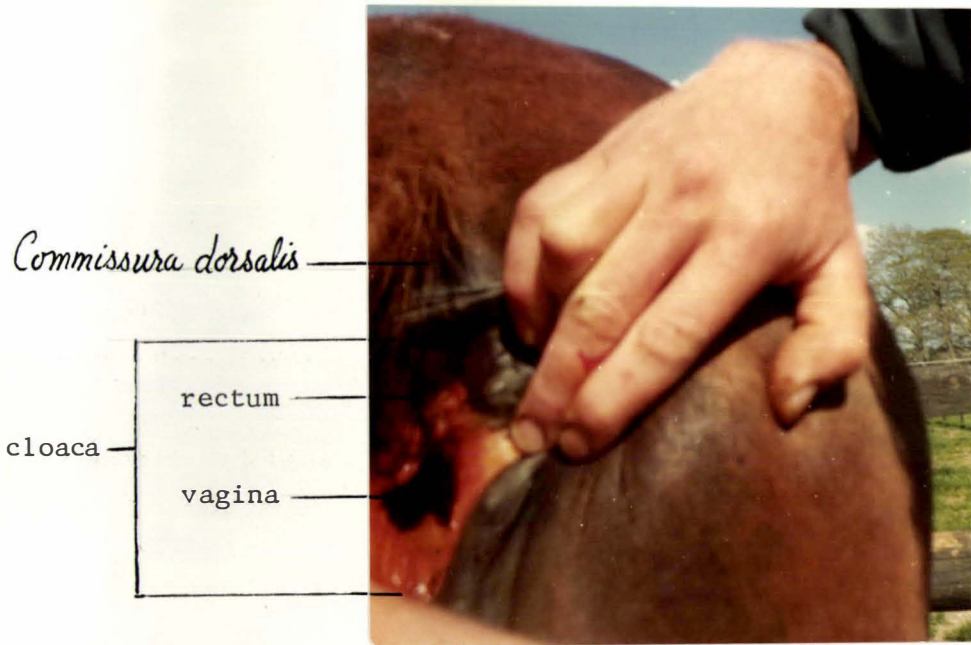
H-47's external genitalia appeared to be normal. During periodic examinations per rectum, since the age of two, H-47's pelvic canal and utero-ovarian structures were found consistently to be normal for her age.

At birth H-47 had a recto-vaginal fistula and atresia ani, and the anal sphincter was situated about 3.75 cm along the ventral surface of the tail. The vaginal opening was normal. The filly was operated on when she was six days old and a continuous rectum and "cloaca" were formed (Figure V.4b). When H-47 was about to turn three, she was again operated on and separate anal and vaginal openings were created. H-47 was reported to have retained some muscular control of defaecation following this surgery.

5.2.3.3. Reproductive history

H-47 was seen exhibiting mild signs of oestrus as a two year old, once late in the breeding season. She was not mated at that time because she was considered to be too immature for breeding and corrective surgery (section 5.2.3.2.) had not yet been performed.

H-47 was teased daily from September to early December, 1979, to which she exhibited no overt signs of oestrus. In early December, 2.5 cc of Prostaglandin "Lutalyse" (Upjohn Pty. Ltd., Auckland) was administered to her, but she still failed to show any signs of heat within the next ten days. According to her veterinarian, examination per rectum revealed ovaries similar to those of a mare still in winter anoestrus. Progesterone withdrawal treatment was commenced using 7.5 gm of "Regumate" (Hoechst, New Zealand Ltd.) administered orally for ten consecutive days. The mare exhibited signs of oestrus four days after cessation of this treatment and she was served. H-47 was diagnosed in foal to that service at 48 days and was reported still in foal at ten months.



5.2.3.4. Behaviour

H-47 was described by her veterinarian, owners and members of the staff as good tempered. She was known to graze freely with other mares. She had not been observed displaying any signs of abnormal sexual behaviour. H-47 was reported to move with less grace than the other mares and had been seen only walking or trotting, never cantering.

5.2.4. Cytogenetic Materials and Methods

5.2.4.1. H-46

Cells were cultured according to the procedures described in Appendix VI. Plasma clot cell cultures were initiated from a skin biopsy obtained under local anaesthesia from the neck. Metaphases analysed from cultured skin had a total elapsed time in culture of less than four weeks.

5.2.4.2. H-47

Cells were cultured according to the procedures described in Appendix VI. Three leucocyte cultures were made over a one year period. Cell cultures were initiated from tissue from the recto-vaginal area. The second finger of the hand in Figure V.4b shows the location of the sample. The sample was immediately placed in transport media and was stored for 24 hours at approximately 5°C, until it could be processed in the laboratory. The cells analysed were from cultures with total elapsed time in culture of less than four weeks. However, some of these cells were from the fourth passage.

5.2.4.3. Evaluation of cells

Polyploid cells were not recorded unless they could be karyotyped. By convention the cells with more than 36 acrocentric chromosomes were considered to contain extra "Y" chromosomes. Metaphases with differential chromosome counts of 63/28 non-acrocentric chromosomes were used to determine an estimated rate of artifactual chromosome loss. However, the probability remained that some of the cells with a normal differential diploid chromosome count of 64/28 non-acrocentrics may have been 65,XXY cells which had lost an acrocentric chromosome during slide preparation.

5.3. Results

5.3.1. H-46

A total of 168 metaphases were evaluated from H-46 (Table 5-1). Twenty-one cells (12.5%) had 65 chromosomes and, by differential count, 28 non-acrocentric chromosomes. Every karyotype analysis showed the extra chromosome to be an acrocentric (Figure V.5). In addition to this presumptive 65,XXY line, 95 metaphases (56.5%) had chromosome counts of 64, of which 28 chromosomes were non-acrocentric. All of these metaphases karyotyped were 64,XX (Figure V.6).

The total estimated rate of artifactual chromosome loss was 12.5%. Therefore, both the one cell determined, on the basis of chromosome morphology, to be 63,XO and the two cells karyotyped as 64,XY (on the basis of non-acrocentric chromosome morphology and the presence of 37 acrocentric chromosomes) were considered to be artifacts rather than evidence of true minor lines.

All cells with greater than 65 chromosomes were from the first cells removed from the primary explant and were therefore not considered to represent in-vitro clone effects. The three cells with 66(28 non-acrocentric) chromosomes karyotyped as 66,XX + two acrocentric chromosomes. However, the scarcity (1.8%) of these cells and their occurrence in only one culture set suggested caution in assuming more than the possibility of such a line in-vivo. There was no pattern to the chromosomes contained either in the three metaphases with 69 chromosomes or the one cell with 92 chromosomes, which were therefore considered to be parts of polyploid cells.

Table 5-1a

The distributions of chromosome counts from H-46's cultured cells.

TISSUES	DISTRIBUTION OF GROSS COUNTS						TOTAL CELLS
	≤ 62	63	64	65	> 65	Polyplloid	
Blood I	2	4	20	3	-	1	30
Skin	11	22	77	18	7	3	138
Total	13	26	97	21	7	4	168
(%)	(7.7)	(15.5)	(57.7)	(12.5)	(4.2)	(2.4)	

Table 5-1b

The distributions of differential chromosome counts from H-46's cultured cells.

TISSUES	DISTRIBUTION OF DIFFERENTIAL COUNTS							TOTAL CELLS	
	≤ 62	63		64		65	> 65		Poly- plloid
	(-)	(28)	(27)*	(28)	(27)	(28)	(**)		
Blood I	2	4	-	20	-	3	-	1	30
Skin	11	17	5	75	2	18	7	3	138
Total	13	21	5	95	2	21	7	4	168
(%)	(7.7)	(12.5)	(3.0)	(56.5)	(1.2)	(12.5)	(4.2)	(2.4)	

*Four 63,XX cells

**Three 66(28) cells, three 69(32) and one 92(34).

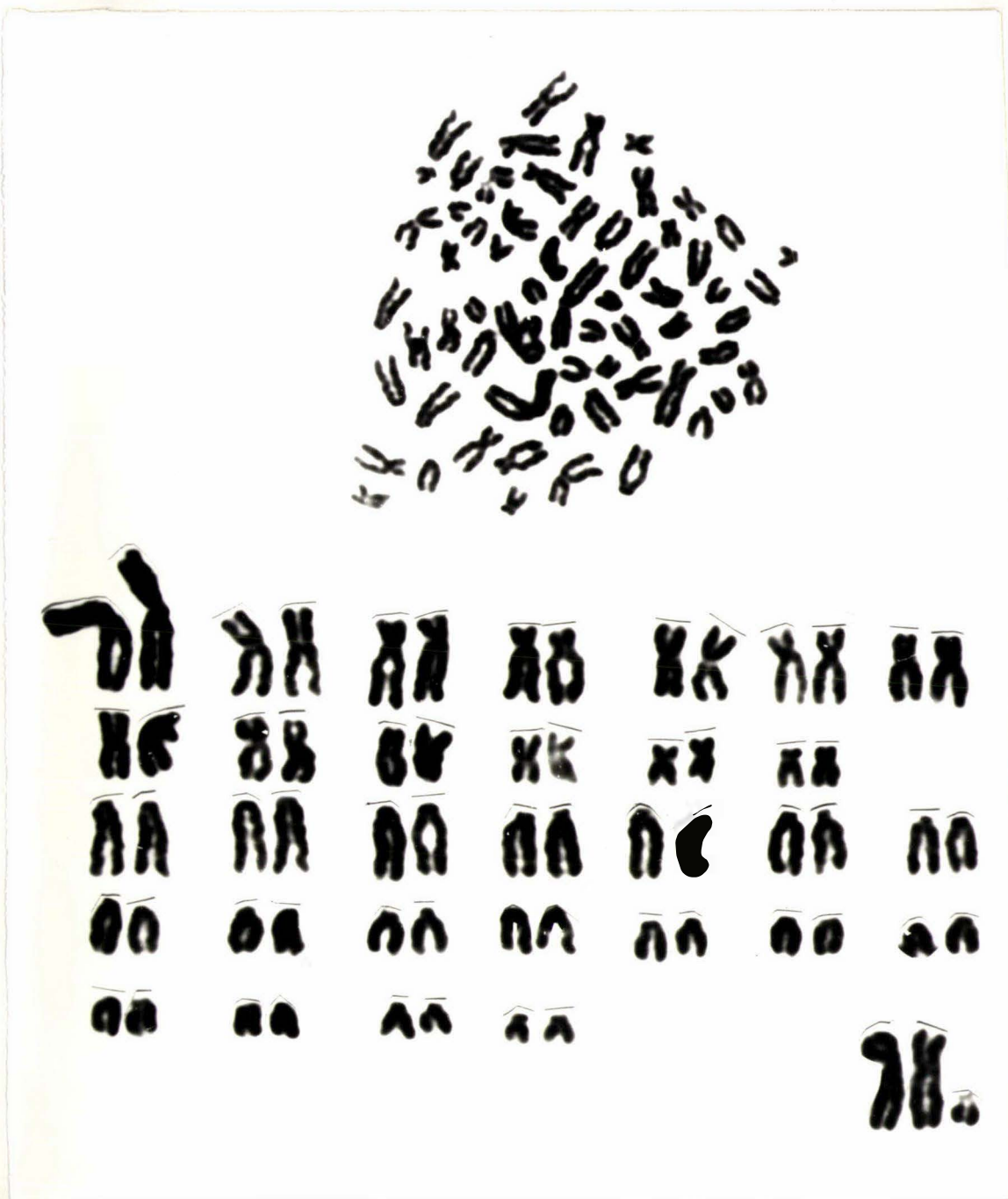


Figure V.5 An example of a cell from H-46 which was designated, by convention, 65,XXY (approx. X 5,000).

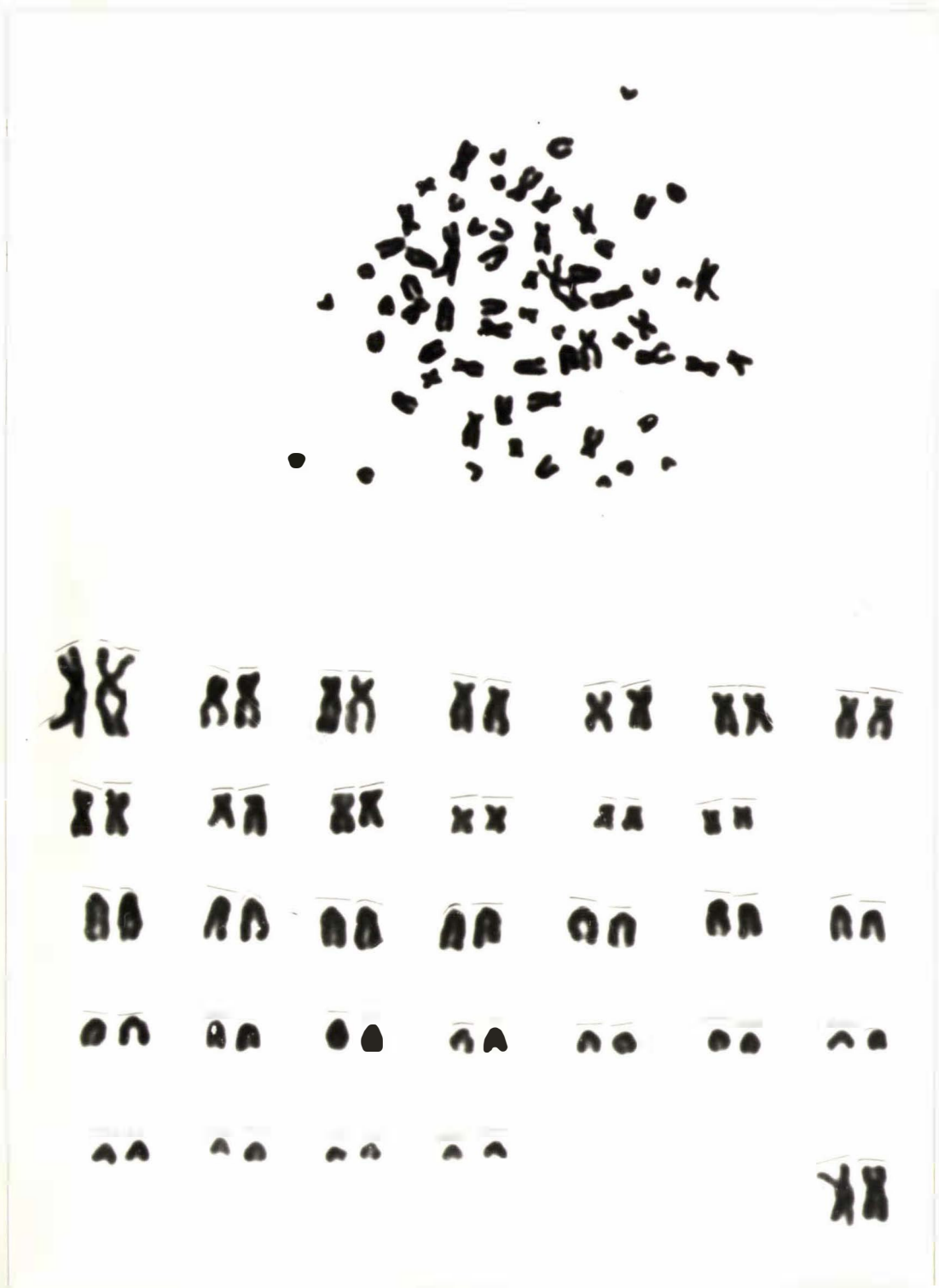


Figure V.6

A normal 64,XX cell from H-46
(approx. X 5,000).

5.3.2. H-47

A total of 185 metaphases were evaluated from H-47 (Table 5-2). Twenty-three cells (12.4%) had 65 chromosomes and a differential count of 28 non-acrocentric chromosomes. Every karyotype analysis showed the extra chromosome to be an acrocentric (Figure V.7). Besides this presumptive 65,XXY line, 112 cells (60.5%) had 64(28 non-acrocentric) chromosomes. All of these karyotyped were 64,XX (Figure V.8).

There was a marked increase in the proportion of cells with 65 chromosomes in passaged cultures (27.0%) over those removed second and third from the explant (4.9% and 5.5% respectively). Passaged cells were from those first removed from the explants and 22.2% of the nine cells evaluated from the first removal had 65 chromosomes. Therefore, neither differential viability in culture nor clone effect was suggested (Benn, 1977). Implied, however, was the possibility that relatively more 65,XXY cells had survived the transfer to in-vitro conditions than 64,XX ones.

The total estimated rate of artifactual chromosome loss was 5.4%. Five of the eight cells with differential counts of 63/27 non-acrocentric chromosomes were 63,XX, so the remaining three were considered to be artifacts. Similarly, the eight cells with 37 acrocentric and 27 non-acrocentric chromosomes might have been considered artifacts (presumably from 65,XXY cells). However, the percentage of 64/27 non-acrocentric metaphases from cultured skin (5.7%) did exceed the 4.8% estimated rate of artifact for cultured skin supporting the possibility of a minor 64,XY line in dermal/epidermal tissues.

The two cells with counts of 66(29 non-acrocentric) chromosomes were from separate skin culture flasks, which raised the possibility that such a line might have existed in-vivo. The other two cells with greater than 65 chromosomes were obviously parts of polyploid cells.

Table 5-2a

The distributions of chromosome counts from H-47's cultured cells.

TISSUES	DISTRIBUTION OF GROSS COUNTS						TOTAL CELLS
	≤ 62	63	64	65	> 65	Polyploid	
Blood I	-	2	17	2	-	-	21
Blood II	-	3	36	5	1	-	45
Blood III	3	1	9	1	-	-	14
Total							
Leucocytes	3	6	62	8	1	-	80
Skin	15	12	58	15	3	2	105
Total	18	18	120	23	4	2	185
(%)	(9.7)	(9.7)	(64.9)	(12.4)	(2.2)	(1.1)	

Table 5-2b

The distributions of differential chromosome counts from H-47's cultured cells.

TISSUES	DISTRIBUTION OF DIFFERENTIAL COUNTS								TOTAL CELLS
	≤ 62	63	64	65	> 65	Poly-ploid			
	(-)	(28)	(27)*	(28)	(27)	(28)	(**)		
Total									
Leucocytes	3	5	1	60	2	8	1	-	80
Skin	15	5	7	52	6	15	3	2	105
Total	18	10	8	112	8	23	4	2	185
(%)	(9.7)	(5.4)	(4.3)	(60.5)	(4.3)	(12.4)	(2.2)	(1.1)	

* Five cells, 63,XX.

** Two cells 66(29) from skin; one 81 and one 70, parts of polyploids.

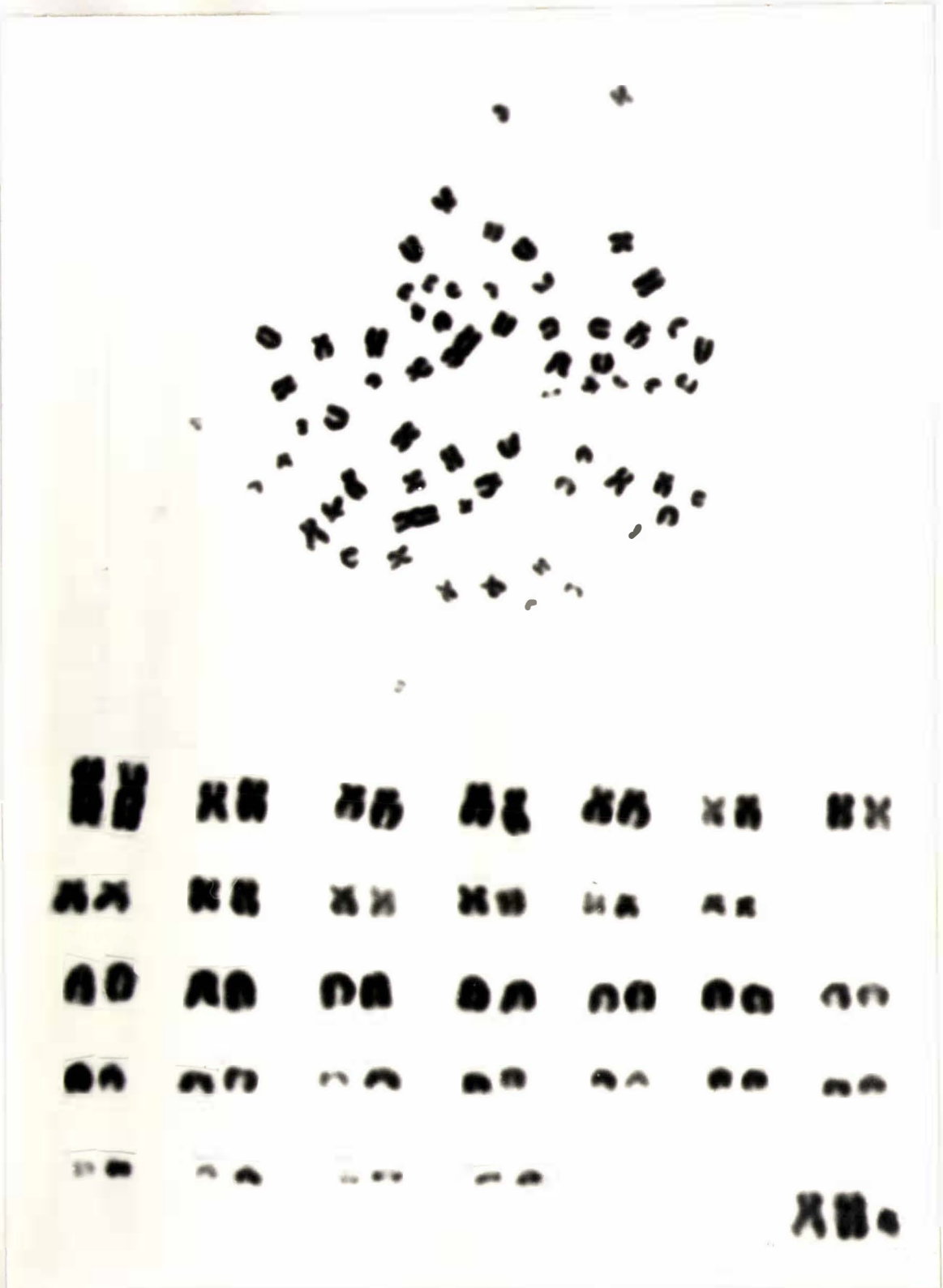


Figure V.7 An example of a cell from H-47 which was designated, by convention, 65,XXY (approx. X 5,000).

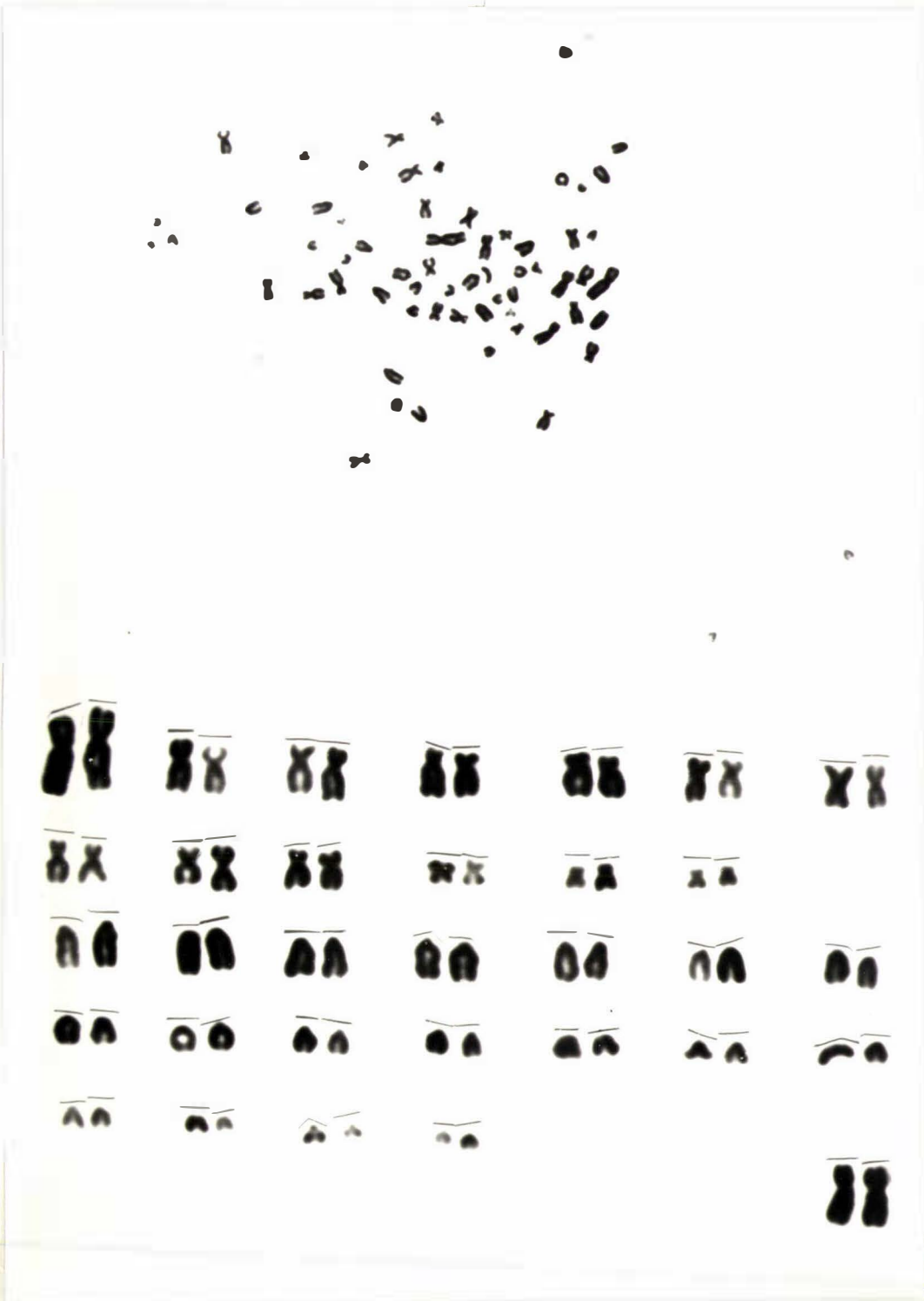


Figure V.8 A normal 64,XX metaphase from H-47
(approx. X 5,000).

5.4. Discussion

H-46 and H-47 had in common an apparent 10-14% XXY mosaic/chimaerism. That estimate, based upon total cells evaluated, may have underestimated the degrees of mosaic/chimaerism in their blood and skin. The proportions of XXY cells became 11-20% when only the cells that had 64 and 65 chromosomes were considered.

According to Ford (1969), the XX/XXY chromosome configuration can arise from a single error. However, that one event can also imply two errors: meiotic non-disjunction in the dam or sire resulting in a 65,XXY zygote and the loss of a Y-chromosome during early cleavage. An additional error must be postulated to achieve XY/XX/XXY mixoploidy, in those cases with a valid XY cell line.

Neither mare's history suggested a predisposition to a cytogenetic abnormality. Both dams had good breeding records and their ages at conception (ten and seven) did not suggest a maternal age effect. On the other hand, both sires were four years old at the times of service, which is young for Thoroughbred stallions. The possible significance of this must await information on other such mosaic/chimaeric mares. The mares did not share a common ancestral Y-chromosome in the four generations traced, and they had only a very slight chance of sharing a common ancestral X-chromosome. The coefficient of relationship between H-46 and H-47, based on incomplete pedigrees, was 0.459 which, on its own, was not thought to be significant.

Aside from H-47's congenital anomalies, nothing was found to suggest that these were unusual Thoroughbred mares. There may well be others like them in the equine population. H-46 and H-47 were the first two non-intersex XX/XXY mares investigated. Neither the extent of their individual mosaic/chimaerism nor the relationship between it and their fertility was established.

H-46 had to be considered "infertile". The prognosis on her ability to carry a foal, based on her severe endometrial hypoplasia, was negative. One could only speculate as to whether that was an acquired condition. That is, whether if she had been allowed (or assisted) to breed when younger she might have conceived. H-46 was probably not sterile because she was reported to have had occasional follicles which were palpable per rectum, and in addition she may have experienced "silent" heats.

There was insufficient information to make an assessment of H-47's fertility. Moreover, since she was young and in her first season, no conclusions should have been drawn as to her ultimate fertility. She was reported to have shown mild oestrous behavior as a two year old and as a three year old was induced, with exogenous hormones to come into season. She was diagnosed as pregnant by both blood test and rectal palpation, and was still in foal at ten months.

Sex-chromosome abnormalities are unlikely to be suspected when the phenotype is normal (Jacobs, 1969). This may have contributed to the lack of reports in the literature of XX/XXY phenotypic females. The majority of XX/XXY females in the literature have displayed some degree of intersexuality (e.g., Dain and Walker, 1979). Conversely, about 17% of "XX" men (female pseudohermaphrodites) have been found to be mosaic/chimaeric for an XXY line (Miro et al., 1979), and both "XX" men and human true hermaphrodites have been shown to carry "Y" genes, if not the Y-chromosome (Wachtel, 1979, 1980). Hermaphrodites containing XXY cells have been reported in humans (Ferguson-Smith, 1966; Bishop, 1972; Opitz et al., 1979), cattle (Dunn et al., 1970), swine (Toyama, 1974) and dogs (Pullen, 1970). That this chromosome configuration occurred in hermaphrodites suggested that it could be found in females of normal phenotype (Ferguson-Smith, 1966; Jacobs, 1969). Since two pregnant true hermaphrodites had been reported (Kim et al., 1979), there was no reason to suppose that all XX/XXY women would be infertile.

Previously reported cases of XX/XXY mosaic/chimaerism in the domestic horse, in line with human reports, have all displayed masculine intersexuality (Bouters et al., 1972, 1975; Fretz and Hare, 1976). Both Dunn et al. (1974) and Basrur et al. (1969, 1970) reported an XY line in addition to the XX and XXY lines, although the described phenotype resembled Bouters et al.'s (1972, 1975) animal. Kodagali (1969) reported an intersex horse, the karyotype of which was not known, which also resembled the above cases. Bornstein (1967) reported two "XX" intersexes but examination of his data suggested the possibility of low level mosaicism for a line with 65 chromosomes in his case II and a line (or lines) with either 65 or 66 chromosomes in his case I. However, in his case I only 19 cells were evaluated and no description was given of the karyotype of the hyperdiploid cells reported (Bornstein, 1967). Insufficient information was supplied by Gerneke

and Coubrough (1970) and Bielański (1977) on their "XX" intersexes, but mosaic/chimaerism might be suspected from the description of the animals.

Studies of freemartin cattle (Vigier et al., 1970; Greene et al., 1977); sheep (Bruère, 1966; Bruère and McNab, 1968; Dain and Tucker, 1970; Dain, 1974) and pigs (Toyama, 1974) have shown a distinct lack of correlation between the degree of masculinization and the proportion of "XY" lymphocytes present. The same situation may or may not hold true for whole body chimaeras. In the XX/XY chimaeras of certain murine strain combinations a preponderance of phenotypic males apparently develops (Mullen and Whitten, 1971; Ohno et al., 1976). A tendency had also been shown for male development in the few XX/XY artificial sheep chimaeras investigated, irrespective of the estimated proportions of XX cells present (Tucker et al., 1974, 1978). Therefore, there was some support for Ohno's (1976) theory that in mammals XX/XY chimaeras tend to develop either testicular like gonads without germ cells (as in freemartins) or functional testes as in murine and sheep artificial whole body chimaeras and bull haemopoietic chimaeras.

Ironically, "XY" mares have been reported to be phenotypically female, sometimes cycling and in one case fertile (Chandley et al., 1975b; Hughes and Trommershausen-Smith, 1976; Kieffer et al., 1976; Trommershausen-Smith et al., 1979; Sharp et al., 1980). Development of functional feminine traits in the presumptive or confirmed presence of H-Y antigen, suggests the presence of hermaphroditism, receptor cell defect, cryptic mosaicism or loss of quorum of H-Y genes (Sharp et al., 1980; Wachtel, 1980). Any or all of those possibilities might exist in a mosaic/chimaera whose sex chromosome constitution was predominantly XX. In other words, the possibility of a genetic disorder, such as testicular feminization, having been masked by the presence of mosaic/chimaerism in H-46 and H-47 cannot be excluded.

Ohno et al. (1976) proposed that only a few cells could disseminate enough H-Y antigen to coat XX cells. The coated XX cells would then collectively act to promote testicular differentiation (Ohno et al., 1976). Such a mechanism would require non-disjunction in only a few unstable but well placed XXY cells relatively late in embryogenesis, or a few strategically located XXY cells capable of sufficient dissemination of H-Y antigen, to explain those masculinized

cases of XX/XXY mosaic/chimaerism reported in the literature. Conversely, the failure of those mechanisms could explain H-46 and H-47's female phenotype. If Ohno *et al.*'s (1976) theory is correct, however, it implies that female development could be more probable than male development in low level equine XX/XXY mosaic/chimaeras.

Neither H-46 nor H-47 showed physical signs of intersexuality. In both ovaries had been palpated *per rectum* and while some ovarian activity had been reported in H-46, H-47 was diagnosed (by blood test) as pregnant 48 days after service. The intersex described by Bouters *et al.* (1972, 1975) tended to a female conformation with a rudimentary vagina, although it had a penis and testes. Both cases described by Bornstein (1967) also tended to female conformation, case I had an enlarged clitoris and case II a penis, although both had a uterus and testes. The case described by Fretz and Hare (1976) had an enlarged clitoris and testes without germ cells.

There were unsubstantiated reports of mild masculine behaviour by H-46. However, strong masculine behaviour was reported in intersex horses by Fretz and Hare (1976), Bouters *et al.* (1972,1975) and Bornstein (1967).

In those equine intersex cases where replicate tissues were studied, the possibility of freemartinism could be eliminated since chorio-vascular anastomosis occurs too late in horses either to influence sexual development or for embryoblast inclusion (Vandeplassche *et al.*, 1970; Bouters and Vandeplassche, 1972). However, the possibility of whole body chimaerism in the absence of genetic markers remained in such cases.

In both H-46 and H-47 leucocytes and cultured cells from skin biopsies were examined and a range of 10-14% 65,XXY cells were found. That is nearly twice the proportion of XXY cells that have been reported in intersex horses with such a cell line. Bouters *et al.* (1972, 1975) examined only laucocytes. Too few cells were evaluated by Bornstein (1967) in his case I and those were only from one tissue (skin). However, in his case II cultured cells from both skin and Müllerian tissue were analysed (47 and 37 cells respectively) (Bornstein, 1967). Fretz and Hare (1976) examined blood and cultured cells from testis and epididymis. In these cases XXY mosaic/chimaerism ranged between 4% and 7%.

The gross developmental differences between reported equine XX/XXY intersexes and H-46 and H-47 might suggest the possibility of

different etiologies for the two non-intersex mares. For example, in the intersex cases an undiscovered or lost XY line (or XXY) might have been predominant at the time of male determination. Alternatively the cells giving rise to mosaicism might have been of different chromosome configurations, i.e., XY, XXY, XXYY.

The event that might have given rise even to the mosaic/chimaerism in H-46 and H-47 may not have happened at the same stage of development in both mares. Also, even if the event occurred at the same time in both mares, the sampling events taking place in precursor cell pools may not have been the same. If the reports of slight behavioural or conformational masculinization of H-46 were accepted, or one accepted a relationship between H-47's congenital defects and her chromosomal constitution, it would seem unlikely that H-46 and H-47 had had similar mosaic/chimaerism at crucial stages of their embryogenesis.

5.4.1. Errors of Development and H-46 and H-47

Developmental errors are found with higher frequency amongst chromosomally abnormal individuals. Bauld *et al.* (1974) reported that 17.8% of human peri-natal and neonate mortalities having congenital malformations also had chromosome abnormalities.

The sinking of the dorsal commissure of the vulva noted in H-46 could have represented an anomaly of development. However, this condition was noted when the animal was moderately old (9 years) and when she was in racing trim, circumstances which can emphasize the condition (Pascoe, 1979). Since H-46 had had no record of pneumovaginitis, a progressive situation associated with aging was implied, rather than a congenital or an inherited abnormality (Pascoe, 1979).

H-47, on the other hand, was investigated for cytogenetic abnormalities because of unequivocal congenital defects, the pattern of which suggested the possibility of an intersex condition. That H-47 was found to be an XX/XXY mosaic/chimaera may have been entirely fortuitous. Conversely, she was the first mare reported with congenital defects and an XX/XXY mosaic/chimaeric condition.

The incidence of atresia ani was reported to be slightly higher in boys than in girls (Bergsma, 1973). However, its incidence was reported to be significantly higher in male lambs (Dennis and

Leipold, 1972), male pigs (Norrish and Rennie, 1968), and was suggested to be higher in bull calves (Leipold *et al.*, 1976). Anal atresia is also a frequently found malformation in severe cases of female pseudohermaphroditism (Overzier, 1963).

The severity of H-47's congenital defects may have related to unknown sex chromosome interactions with developmental mechanisms. Disturbances in the division of the embryonic cloaca and in the formation of the cloacal membrane and faulty or arrested migration of the urorectal septum have been implicated in both atresia ani and recto-vaginal fistulae formation (Arey, 1962; Overzier, 1963; Nieberle and Cohrs, 1967; Rawlings and Capps, 1971; Harris *et al.*, 1979). Cases of atresia ani with recto-vaginal fistula have been reported in Buffalo (Marler *et al.*, 1977), dog (Rawlings and Capps, 1971), cattle (Nair, 1972; Leipold *et al.*, 1976), squirrel monkey (Harris *et al.*, 1979) and sheep (Gerneke, 1967).

The temporal developmental pattern of the anal sphincter relative to that of the urogenital sinus (Arey, 1962; Marrable, 1971) suggested that the displacement of H-47's anal sphincter could be considered a separate congenital defect. This was thought probably to have resulted from the abnormal embryonic environment established by the other defects, including possible abnormal skeletal development patterns.

The etiology of atresia ani is unknown (Dennis and Leipold, 1972; Huston *et al.*, 1977). Although genetically produced there may be an influence by exogenous environmental factors (Nieberle and Cohrs, 1967; Smidt, 1972; Harris *et al.*, 1979). In humans most anorectal anomalies are considered chance occurrences, although both environmental factors and in some families, an autosomal recessive mode of inheritance have been implicated (Bergsma, 1973; Harris *et al.*, 1979). In swine, there were strong indications that there was a polygenic component with high penetrance in the inheritance of atresia ani (Norrish and Rennie, 1968; Smidt, 1972).

Atresia ani is occasionally seen in horses (Huston *et al.*, 1977). However, it is one of the more common congenital defects in humans, occurring in one in approximately 4,500 to 5,000 live births (Overzier, 1963; Bergsma, 1973; Morson, 1978; Harris *et al.*, 1979). It is more common in swine, appearing in 0.4 to 0.6% of births (Norrish and Rennie, 1968; Smidt, 1972). Atresia ani was reported

in sheep (Gerneke, 1967; Dennis and Leipold, 1972) and was said to have been involved in up to one in 69 perinatal lamb mortalities (Dennis and Seddon, 1971). It is a relatively rare congenital defect in cattle (Greene et al., 1973; Leipold et al., 1976). However, in dogs it was suggested to be under-reported (Rawlings and Capps, 1971), and this may be true of other domestic animals.

Chromosome anomalies had not been found in association with the studied cases of atresia ani in either swine (Vogt, 1967) or sheep (Gerneke, 1967). However, possible mosaicism was not investigated, and the possibility could not be excluded in the four animals evaluated. In humans, atresia ani is not considered to be related to chromosome abnormalities (Overzier, 1963). However, some autosomal deletion syndromes are associated with the condition in humans (Bergsma, 1973) and in cattle a ring autosome was associated with the condition (Lajda et al., 1976). Atresia ani is a component of several recognized human syndromes as well as "fused" mutation in mice (Harris et al., 1979). It is usually found in association with other congenital defects (Dennis and Leipold, 1972; Bergsma, 1973; Leipold et al., 1976; Rawlings and Capps, 1977).

Spinal anomalies in domestic animals usually involve the caudal region (Nieberle and Cohrs, 1967) and are common complications in anorectal malformations in humans (Bergsma, 1973). In humans, various skeletal anomalies have been reported in connection with all chromosome abnormalities, however no specific relationships have been discovered. Amongst those individuals with sex chromosome dysmorphism, skeletal malformations of various kinds, including underdeveloped sacrum and coccyx and fusion of spinal or cervical vertebrae, are frequently reported (Polani, 1961; Overzier, 1963; Morishima and Grumbach, 1968; Hsueh et al., 1978). Skeletal malformations are common in women with gonadal dysgenesis, and comprise part of the Turner syndrome (Turner, 1938).

Reduction in the number of vertebrae resulting in shortening of the tail may be a phenomenon of chance or may be inherited (Nieberle and Cohrs, 1967). Short tail has been reported in association with atresia ani in squirrel monkey's (Harris et al., 1979) and cattle (Greene et al., 1973). For that matter, tail-lessness, due to agenesis of the tail bud (Nieberle and Cohrs, 1967), was reported in association with nine of sixteen cases of cattle with atresia

ani (Greene et al., 1973; Leipold et al., 1976).

Therefore, it was considered probable that intensive anatomical studies of H-47 would disclose anomalies and possibly confirm her veterinarian's opinion that she possessed skeletal anomalies.

5.4.2. Summary

H-46 and H-47 were 64,XX/65,XXY mosaic/chimaeras with no physical signs of intersexuality. H-46 was presented for cytogenetic analysis because her trainer thought she had masculine traits. Only after her initial diagnosis as a mosaic/chimaera was her "infertility" seriously questioned. H-46's severe endometrial hypoplasia suggested that she would be incapable of carrying a foal to term and justified a diagnosis of "infertile". H-47 was a young maiden mare investigated for cytogenetic abnormalities because she had been born with defects of development of the caudal axis. Following treatment and therapy, H-47 was served and was diagnosed as in foal. However, there was insufficient information to assess her fertility.

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H-27, AN ENIGMA

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CHAPTER VI

H-27, AN ENIGMA6.1. Introduction

H-27 was an apparently normal mare admitted to the Large Animal Unit at Massey University for investigation of chronic endometritis. Coincidentally H-27 was admitted at the same time as a mare suspected of being an XO/XX mosaic (Bruère et al., 1978), and blood samples were obtained from H-27 for control lymphocyte cultures. It was the consistent failure of H-27's lymphocyte cultures to yield sufficient metaphases for analysis that suggested she may have had an abnormal karyotype.

An initial cell culture, from a skin biopsy, provided three cells which suggested the possibility of a minor 66,XXXX cell line. Post-mortem sex-chromatin studies of the central nervous system confirmed the presence of two inactive X-chromosomes in some of her neurons. However, cell cultures from repeat skin biopsies and from tissues obtained at autopsy were suggestive of a general mitotic instability in-vitro. They also raised the possibility of either a polyploid cell line in-vivo or a tendency to polyploidy in-vitro.

In view of the apparent mitotic instability in-vitro of H-27's cells, and in an attempt to both understand why the lymphocyte cultures from H-27 failed to grow and quantify the deficit, lymphocyte transformation tests were performed. These verified that her

lymphocytes were functionally defective in phytohaemagglutinin stimulation systems. Since depressed lymphocyte transformation is evidence of anomalies of the immune system, this lead to speculation about H-27's immune status relative to both her karyotype and her infertility.

Access to H-27 for cytogenetic studies was limited. However, it was possible to observe the mare over extended periods and her records were made available. It was also possible to participate in her autopsy and to obtain tissue samples at that time.

6.2. Materials and Methods

6.2.1. H-27

6.2.1.1. Description

Sex:	female
Foaled:	1971
Breed:	Thoroughbred
Colour:	chestnut
Age of sire at birth:	10 years
Age of dam at birth:	6 years

6.2.1.2. General information

H-27 was admitted to the Large Animal Unit at Massey University, Faculty of Veterinary Science, on the 21st of February, 1978. At the time of admission she was a normal sized six year old mare, in good condition except for chronic endometritis.

6.2.1.3. Pre-admission history¹

At ages four and five, H-27 had been sent to different studs. Both reported that she had "failed to hold". Neither stud reported any history of infection. She arrived at the third stud, as a six year old, where she exhibited profuse purulent vaginal discharge. She showed oestrus and was served to a follicle on the right ovary following successful treatment for pyometra. Three weeks later she returned to season and was served again. Five weeks after the second service she again developed pyometra. The condition was treated but she was referred to the Faculty of Veterinary Science, Massey University.

6.2.1.4. History at Massey University²

H-27 never showed a regular pattern of oestrus. She did come into heat naturally from time to time, and oestrus could be induced during prolonged dioestrus using prostaglandins. Three

¹ Sources: Initial referral letters and case records.

² Source: Report to owner(s), Reproduction Unit, Massey University, June, 1979.

endometrial biopsies showed progressive deterioration, from sub-acute toward chronic (with some glandular fibrosis) endometritis. The animal never exhibited pyometra, even following service; however, recurrent low grade haemolytic streptococci were isolated from uterine swabs.

The mare was thought to have conceived in December, 1978; however six weeks later she came into oestrus. She was not pregnant in April, 1979, despite having been run with the stallion for six months. The prognosis for successful pregnancy, based on endometrial deterioration and clinical pattern, was considered to be poor. In accordance with the terms of her referral the mare was destroyed.

6.2.1.5. Behaviour

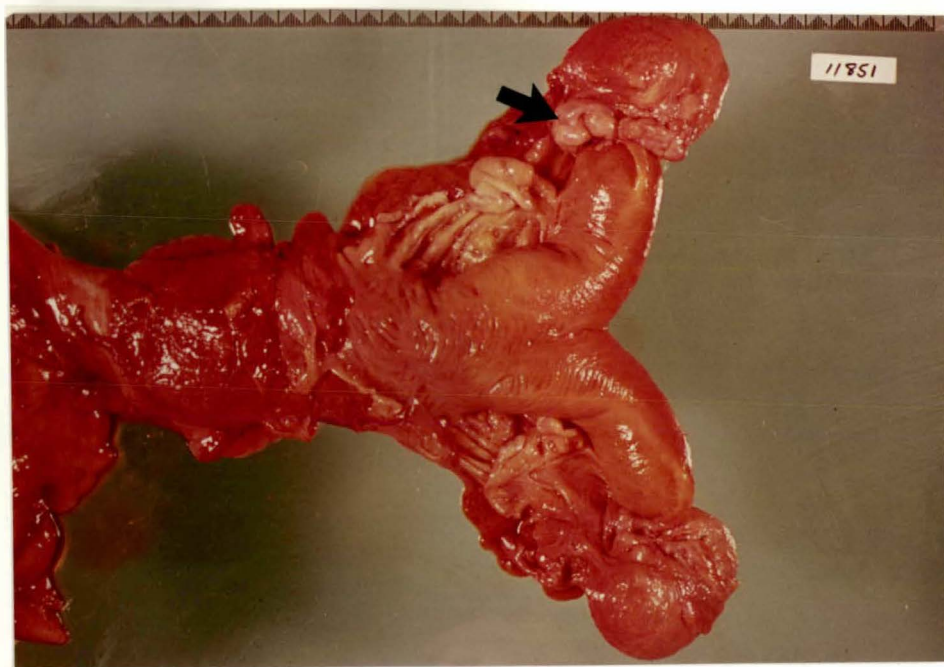
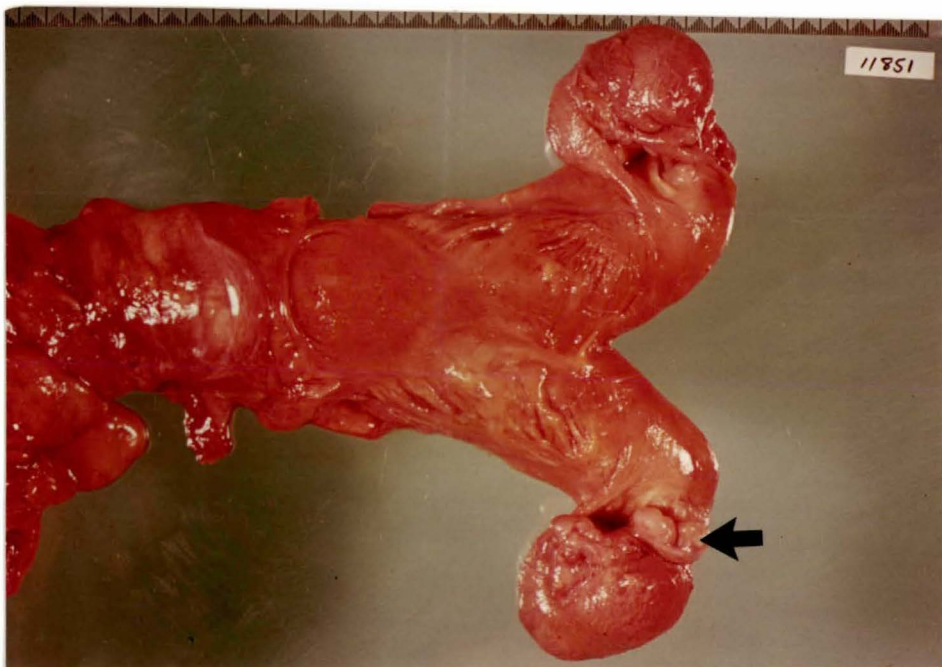
H-27 was an unbroken mare and in general she seemed to be indifferent to humans. When she was first put into a paddock with a stallion at Massey, he bit and kicked her enough for her to require treatment. Thereafter, there were no problems between them until an attempt was made to integrate H-27 into the Massey herd of horses. At that time either the stallion or the other mares savaged her. Following this she was reported to be usually solitary and on the fringes of the herd whenever observed during the Animal Behaviour classes (R. Holmes, pers. comm.).

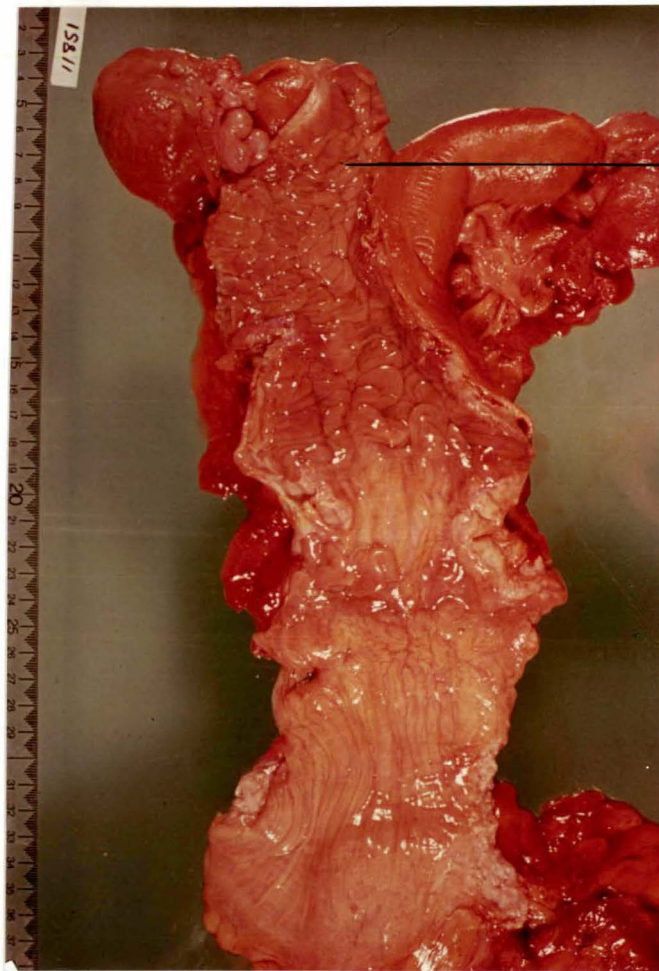
6.2.1.6. Post-mortem report³

H-27 was destroyed on 5th June, 1979, when she was just under eight years of age. At autopsy she was found not in oestrus and not pregnant. Post-mortem findings are given in detail in Appendix IV.

H-27's internal genitalia (Figures VI.1 and VI.2) were essentially normal. The uterus appeared to be smaller than normal (Figure VI.2), but not unusually so for a mare which was neither pregnant nor in oestrus at the time of death, and which had never had a foal. The right Fallopian tube was enlarged (5 mm in diameter). It ended "blindly" at the ovary and no fimbriae were present. Five

³Sources: Post-mortem Report number 11851 and Dr. M. Alley,
 Department of Veterinary Pathology, Massey University.





right *Cornua uteri*
(uterine horn)

Corpus uteri
(uterine body)

Cervix uteri
(cervix)

vagina

fluid filled cysts (5 mm in diameter) were present either in the ovarian fossa or paraovarian region. The ovaries were both 5 cm in diameter. The right ovary contained several developing follicles (0.5 to 1 cm in diameter). The left ovary contained a corpus luteum (2 cm in diameter).

The post-mortem findings included exudative dermatitis, sub-acute endometritis, paraovarian cysts, subcutaneous oedema and moderate alveolar emphysema. A well differentiated papilloma was also present on the left third eyelid.

6.2.2. Cytogenetic Materials and Methods

6.2.2.1. Cultured cells

The procedures used in culturing cells for chromosome preparations are described in Appendix VI. Due to a temporary shortage of fetal bovine serum, three flasks of the Skin I culture were initiated with growth media supplemented with homologous serum. No distinction was made between these two treatments in the analysis of the chromosome preparations.

The chromosome preparations analysed from Skin I, II and III, and uterus cell cultures were from plasma clot cultures. Cells from both trypsin digestion and plasma clot techniques were analysed from kidney, spleen and muscle cultures. Total elapsed time in culture for the epithelial-like cells from uterus cell cultures was six to nine weeks. All other cells had less than four weeks in culture before they were harvested.

Polyploid cells were not recorded unless they could be karyotyped. To estimate actual rates of polyploidy, the first 100 cells from slides from skin I, skin III and muscle cell cultures were classified, by appearance, as less than diploid, approximately diploid and polyploid. Exact chromosome counts were not attempted except to verify the accuracy of classification, so that metaphases of poor quality could be included in the estimation of overall rates of polyploidy.

Cell culture techniques used for lymphocyte transformation tests are given in section 6.2.2.3. and Appendix VI.

6.2.2.2. Sex-chromatin investigations

The procedures used for sex-chromatin studies of the cerebellum and spinal cord of H-27 are described in Appendix VI.

6.2.2.3. Lymphocyte transformation test

The system of Ramadass (1980) was used to micro-culture lymphocytes and measure the rate of transformation, as is described in Appendix VI. The micro-culture plate was set up in a modified Roman Square, utilizing a series of phytohaemagglutinin (PHA) concentrations and incorporated three replicates for each concentration. A facsimile of the culture plate showing the experimental design is given below (Figure VI.i). Columns 4, 8, and 12 were controls consisting of media and lymphocytes without mitogen to give background levels of radiation. PHA concentrations are given in μg , (G) = control horse, S = serum, L = lymphocytes.

Figure VI.i

	1	2	3	4	5	6	7	8	9	10	11	12
(H-27)L (H-27)S	1	1	1	0	5	5	5	0	10	10	10	0
(H-27)L (H-27)S	25	25	25	0	50	50	50	0	100	100	100	0
(H-27)L (G)S	1	1	1	0	5	5	5	0	10	10	10	0
(H-27)L (G)S	25	25	25	0	50	50	50	0	100	100	100	0
(G)L (G)S	1	1	1	0	5	5	5	0	10	10	10	0
(G)L (G)S	25	25	25	0	50	50	50	0	100	100	100	0
(G)L (H-27)S	1	1	1	0	5	5	5	0	10	10	10	0
(G)L (H-27)S	25	25	25	0	50	50	50	0	100	100	100	0

6.3. Results

6.3.1. Results of Cytogenetic Investigations

A total of 550 metaphases from cultured cells from H-27 were evaluated (Table 6-1). Of these 334 (60.7%) had a normal diploid differential count of 64/28 non-acrocentric chromosomes. There was no suggestion that H-27 was mosaic/chimaeric for either a 63,X0 or an hyperdiploid cell line. There was, however, the suggestion of mitotic instability in-vitro, and the possibility of a polyploid line in-vivo.

In addition 566 neurons from H-27's central nervous system were evaluated for sex-chromatin. Some cells were found with two sex-chromatin bodies, indicating the presence of cells with two inactive X-chromosomes (Figure VI.3a).

6.3.1.1. Cultured cells

The results of cytogenetic analysis of chromosome preparations from H-27's cultured cells are given in Table 6-1. Lymphocyte cultures consistently failed to yield sufficient metaphases for cytogenetic analysis, and the ones obtained were of relatively poor quality. The results shown for leucocyte cultures were the combined results from seven cultures, out of approximately fourteen attempts, over an 18 month period.

Of the 550 metaphases evaluated from H-27, 60 (10.9%) had 63 chromosomes. However, only 13 (2.4%) of these had differential counts of 63/27 non-acrocentrics and only one of those (Figure VI.4) was 63,X0. Therefore, there was no suggestion of a 63,X0 cell line. Twenty-four metaphases (4.4%) had counts of more than or equal to 65 chromosomes. There did not seem to be a pattern to these cells (Table 6-2) and a specific hyperdiploid mosaic/chimaeric cell line was not thought to have been demonstrated.

Fifty-nine (10.7%) of the cells evaluated were polyploid. That figure underestimated the amount of polyploidy since polyploid metaphases seldom had good enough morphology to warrant recording. However, all cell cultures, especially those initiated from skin biopsies, seemed to have an excess of hyperdiploid and hyperploid cells. The estimate of true hyperploidy in Skin III and Muscle

Table 6-1a

The distributions of chromosome counts from H-27's cultured cells.

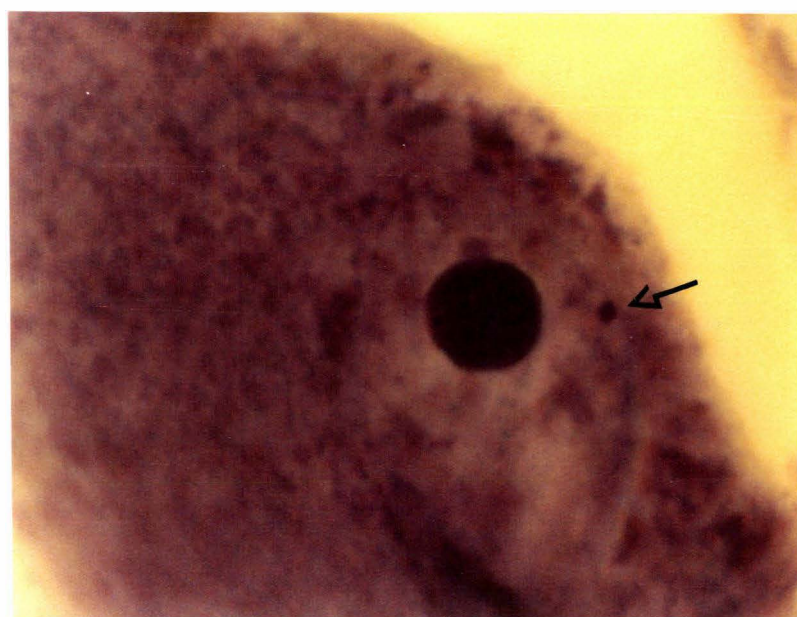
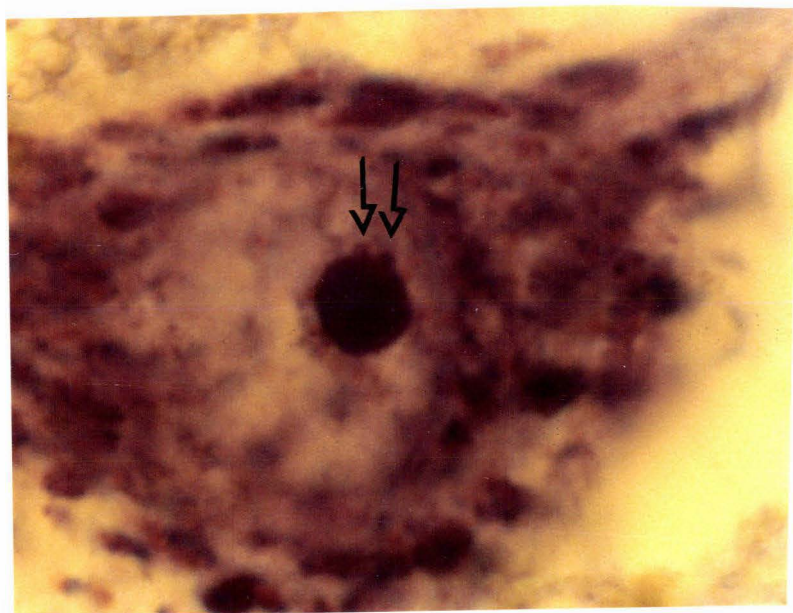
TISSUE	DISTRIBUTION OF GROSS COUNTS						TOTAL CELLS
	≤ 62	63	64	65	>65	Polyploid	
Leucocyte	5	5	29	1	-	-	41
Skin I	11	10	30	-	5	17	73
Skin II	12	6	47	3	3	12	83
Skin III	11	15	67	2	1	9	105
Kidney	13	9	45	-	1	5	73
Spleen	6	6	45	1	3	2	63
Muscle	9	6	58	-	2	2	77
Uterus	5	3	13	-	2	12	35
Total	72	60	334	7	17	59	550
(%)	(13.1)	(10.9)	(60.7)	(1.3)	(3.1)	(10.7)	

Table 6-1b

The distributions of differential chromosome counts from H-27's cultured cells.

TISSUE	DISTRIBUTION OF DIFFERENTIAL COUNTS								TOTAL CELLS
	≤ 62	63		64		65	>65	Poly-ploid	
	(-)	(28)	(27)*	(28)	(-)	(*)	(*)		
Leucocyte	5	5	-	29	-	1	-	-	41
Skin I	11	9	1	30	-	-	5	17	73
Skin II	12	4	2	47	-	3	3	12	83
Skin III	11	12	3	67	-	2	1	9	105
Kidney	13	5	4	45	-	-	1	5	73
Spleen	6	3	3	45	-	1	3	2	63
Muscle	9	6	-	58	-	-	2	2	77
Uterus	5	3	-	13	-	-	2	12	35
Total	72	47	13	334	-	7	17	59	550
(%)	(13.1)	(8.5)	(2.4)	(60.7)	(0)	(1.3)	(3.1)	(10.7)	

* See Table 6-2.



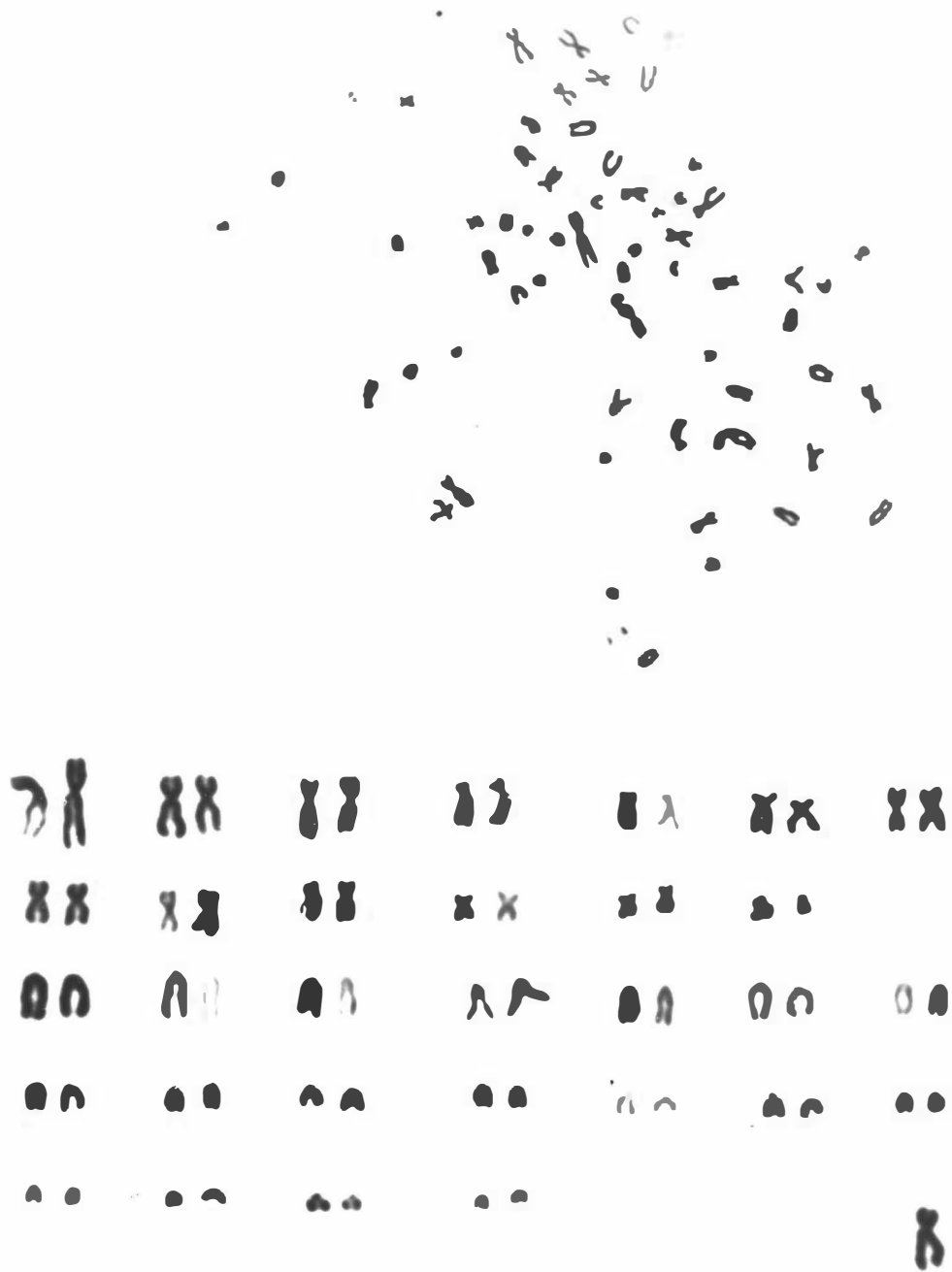


Figure VI.4 The only 63,X0 cell found in H-27's cultured cells (approx. X 5,000).

Table 6-2

Summary of recorded abnormal differential chromosome counts from cultured cells from H-27.

TISSUES	CHROMOSOME COUNTS																				TOTAL CELLS		
	≤ 62	63			65		66		67	68	69	73		77	86	92	96	98	Polyploid				
	(-)	(28)	(27,XX)	(27,XO)	(28)	(29)	(28)	(30)	(27)	(29)	(27)	(31)	(29)	(31)	(32)	(34)	(37)	(38)	(43)	(4N)		(8N)	
Skin I	11	9	1	-	-	-	-	3	1	-	-	1	-	-	-	-	-	-	-	17	-	43	
Skin II	12	4	2	-	2	1	1	-	-	-	-	1	-	-	1	-	-	-	-	12	-	36	
Skin III	11	12	3	-	1	1	-	-	-	-	-	-	1	-	-	-	-	-	-	8	1	38	
Kidney	13	5	4	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	4	1	28	
Spleen	6	3	2	1	1	-	-	-	-	-	1	-	-	1	-	-	-	-	1	2	-	18	
Muscle	9	6	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	1	-	2	-	19	
Uterus	5	3	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1	-	-	12	-	22	
Total	67	42	12	1	4	2	2	3	1	1	1	2	1	1	1	1	1	1	1	57	2	204	
(%)	(32.8)	(20.6)	(5.9)	(0.5)	(2)	(.98)	(.98)	(1.5)	(.5)	(.5)	(.5)	(.98)	(.5)	(.5)	(.5)	(.5)	(.5)	(.5)	(.5)	(.5)	(.5)	(27.9)	(.98)
	'Broken' diploids or polyploids 59.8%				Hyperdiploid cells 5.9%							Parts of polyploid cells and polyploids 34.3%											

Note: All cells with 64 chromosomes had 28 non-acrocentric ones.

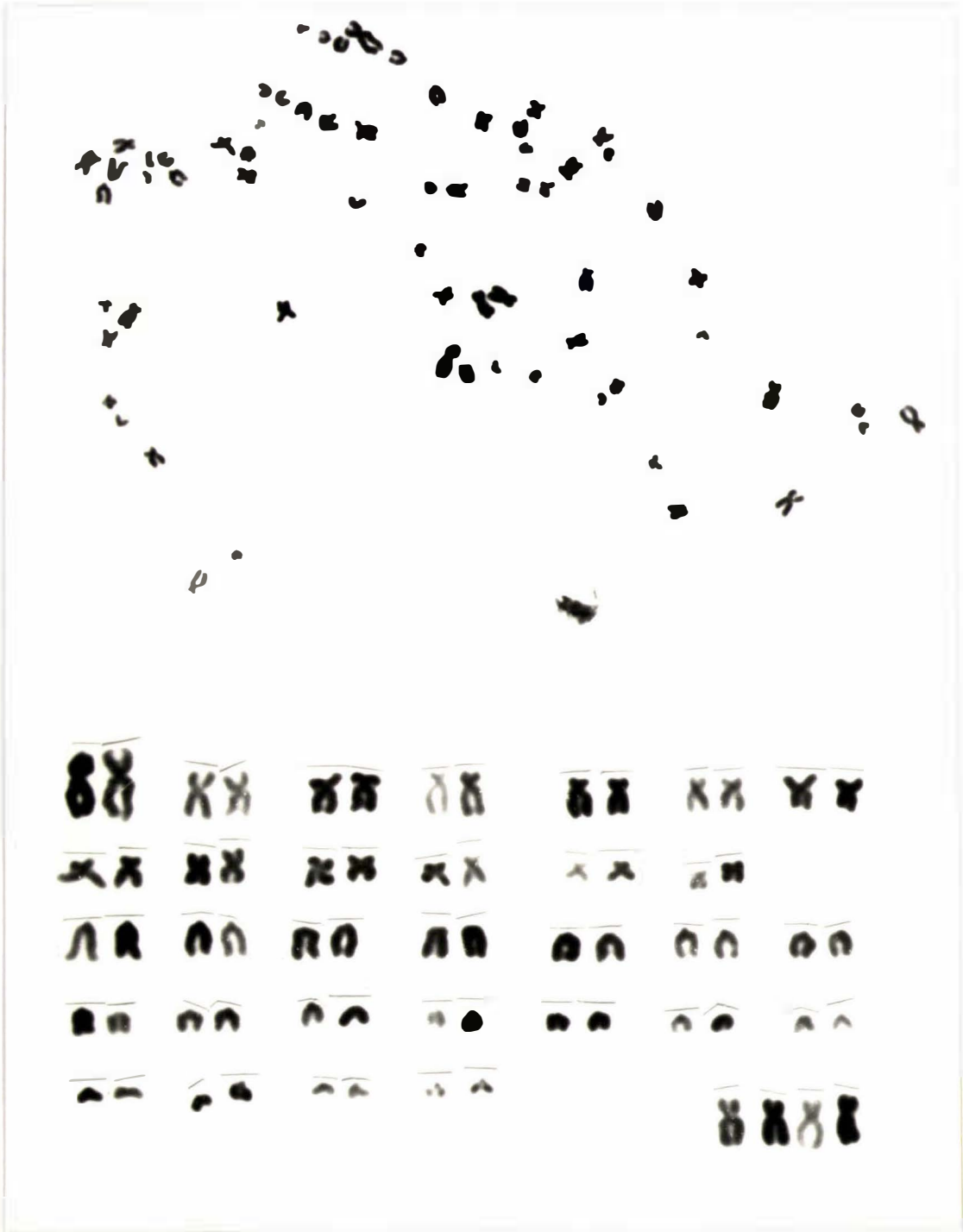


Figure VI.5 One of the three cells which suggested, initially, that H-27 might have a 66,XXXX cell line (approx. X 5,000).



Figure VI.6 One of the subsequent metaphases with 66 chromosomes, suggesting that H-27's cells were mitotically unstable in-vitro (approx. X 5,000).

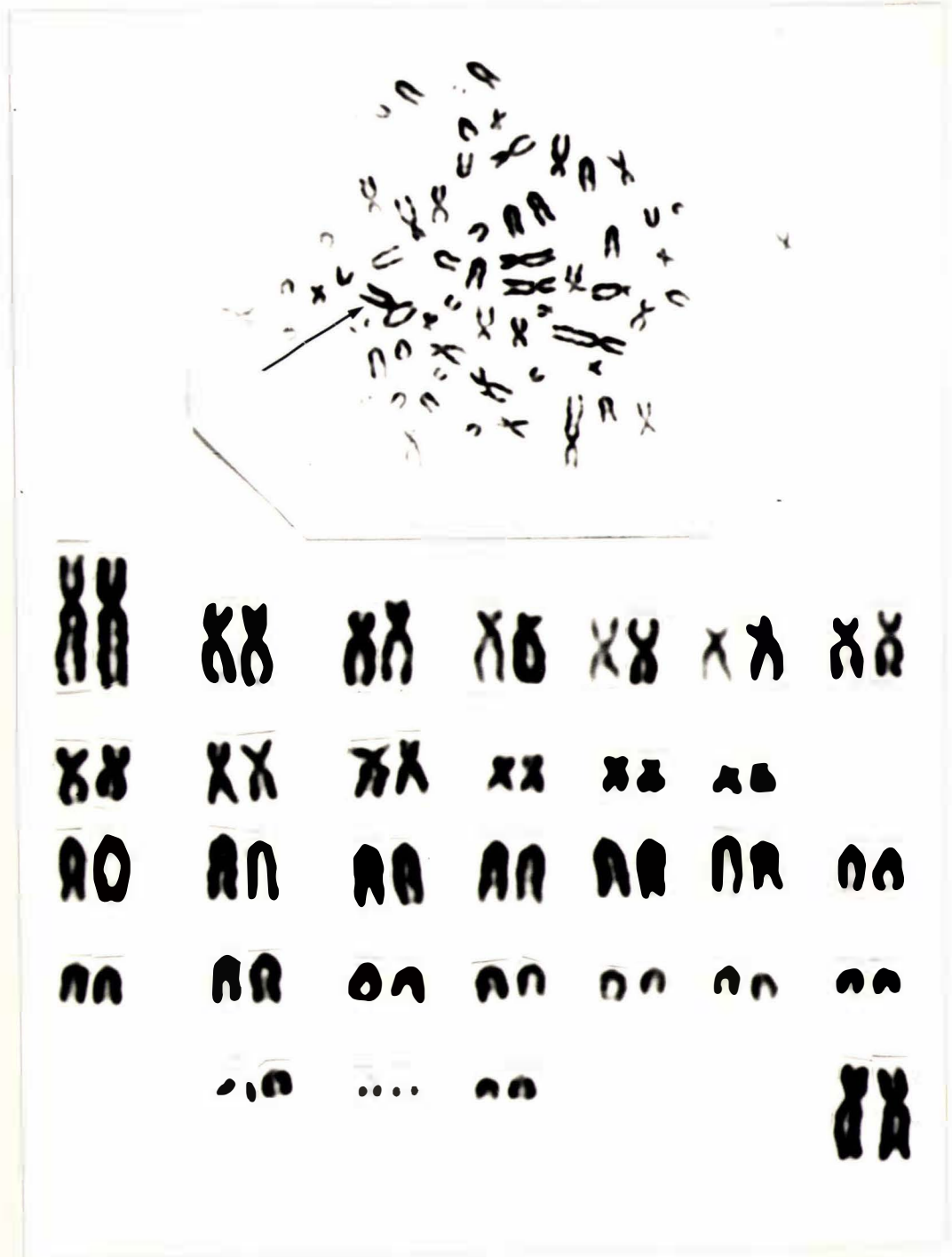


Figure VI.7 An example of a 62,XX cell. This could be the complement of a hyper-diploid cell, as well as an example of chromosome loss during slide preparation. Alternatively, what appears to be two large acrocentrics (arrow) may actually be an abnormal arrangement involving up to four chromosomes. (Approx. X 5,000.)



Figure VI.8 An example of an abnormal chromosome arrangement seen in two of H-27's cultured cells (X 4,250). These arrangements were discussed in Chapter III.



Figure VI.9 An example of endoreduplication in H-27's cultured cells (X 9,500). Signs of endoreduplication were comparatively common.

cell cultures was 33% and in Skin I culture 25%. When broken cells were included, that is cells which could be either part of polyploids or part of diploid cells, a 1:1 ratio was estimated between diploid and broken cells in all three test samples. This indicated either a consistently high rate of polyploidy or a high rate of technical artifact leading specifically to broken cells.

The high proportion of polyploid cells estimated to be present in the cell cultures, as well as the proportion recorded, suggested either that perhaps H-27's cells were particularly sensitive to in-vitro conditions influencing towards mitotic instability, or that a hyperploid mosaic/chimaerism existed in-vivo.

6.3.1.2. Sex-chromatin investigations

Sex chromatin was examined from sections of the cervical (segments upper and middle), thoracic, lumbar and sacral regions of the spinal cord and from the cerebellum. Table 6-3 provides the numbers and distribution of sex-chromatin bodies found.

Blue (1976) observed no neurons with two sex-chromatin bodies during his study of sex-chromatin in a normal mare. However, 1.6% of the neurons examined from H-27 contained two sex-chromatin bodies. This might have indicated neuronal mosaic/chimaerism for cells containing three X-chromosomes, cells with two inactive X-chromosomes, or the presence of polyploid neurons (Barr, 1966; Bühler, 1977). However, there was no suggestion from H-27's cultured cells that she had a triple-X type (i.e., XXX, XXXY, XXXYY) cell line. Furthermore, it is extremely unlikely that a 64,XX cell with two inactive X-chromosomes, in effect a null-0 cell, could be viable even in the central nervous system. Therefore, the most probable explanation for the neurons which displayed two sex-chromatin bodies, was that H-27 contained neurons with either 3N or 4N chromosomes.

The average incidence of neurons with one or more sex-chromatin bodies was 64.5%. This was less than the average of 69% reported by Blue (1976) for a normal 64,XX mare. The difference was almost totally due to an excess (difference of 16.3%) of sacral region neurons without sex-chromatin bodies. However, 13.3% of the sex-chromatin bodies in the sacral region of Blue's (1976) normal mare were located on the nuclear membrane, whereas none were in H-27. This suggested that a difference between these animals, for example

Table 6-3

The numbers and distributions of sex-chromatin bodies in the central nervous system of H-27.

REGION OF NERVOUS TISSUE	NUMBER OF SEX CHROMATIN BODIES			TOTAL CELLS PER REGION	POSITIONS OF SEX CHROMATIN WITHIN THE NUCLEUS				
	0	1	3		One body		Two bodies		
					P.N.	NUC.	1-P.N.	2-P.N.	2-NUC.
Cervical	31	67	2	100	62	5	1	1	0
Mid-cervical	37	63	0	100	55	8	0	0	0
Thoracic	54	123	1	178	114	9	0	1	0
Lumbar	74	123	3	200	107	16	1	2	0
Sacral	95	102	3	200	82	20	0	3	0
Cerebellum	21	74	5	100	73	1	0	5	0
TOTAL	312	552	14	878	493	59	2	12	0
(%)	(35.5)	(62.9)	(1.6)		(87.1)	(10.4)	(0.35)	(2.1)	(0)

P.N. = peripheral to the nucleolus
NUC. = nucleoplasm

death trauma, could have biased the comparison. On the basis of these sex-chromatin results, however, the possibility of either a minor XO line or cells with two active X-chromosomes could not be disregarded.

Analysis of the data in Table 6-3 was performed using a R x C table for attribute data with more than one degree of freedom (Snedecor and Cochran, 1973).

No significant difference was found within segments of sections from the cervical region. The χ^2 for the numbers of sex-chromatin bodies present (cervical segments combined) was probably significant ($P = \infty$), $\chi^2 = 32.333$, d.f. = 8. Thirty-nine and forty-seven percent of the departure from independence was contributed by the sacral region and cerebellum respectively. However, the cytogenetic significance of this result was obscured by the statistical necessity to consider such a result null and void. Partitioning the components of the departure from independence, the greatest contribution was from an excess of sacral neurons without sex-chromatin bodies ($\chi^2 = 8.057$), a shortage of cerebellum neurons without sex-chromatin bodies ($\chi^2 = 7.33$), and an excess of cerebellum neurons ($\chi^2 = 5.94$) with two sex-chromatin bodies.

The χ^2 derived from analysis of the location of the sex-chromatin was highly significant, $P > 0.016$ ($\chi^2 = 29.2449$, d.f. = 15). The contribution of sacral neurons with one sex-chromatin body in the nucleoplasm ($\chi^2 = 7.491$), cerebellar neurons with two sex-chromatin bodies peripheral to the nucleolus ($\chi^2 = 6.600$) and with one sex-chromatin body in the nucleoplasm ($\chi^2 = 6.356$) accounted for 69.9% of the departure from independence.

Figure VI.1a and 1b presents examples of sex-chromatin found in H-27's neurons.

6.3.2. Lymphocyte Proliferation Test

H-27 showed a distinctly lower lymphocyte transformation rate, which was 0.5 to 0.33 that of the control horse. Since this low rate occurred both against her own (homologous) serum and the normal horse's (autologous) serum, it was her lymphocytes, not serum factors, that were defective.

The results from the lymphocyte proliferation trial reported are given in Table 6-4. Three attempts were made, of which the one

reported was the third. Insufficient numbers of lymphocytes were isolated on the first attempt for a complete experiment as designed. On the second attempt, insufficient cells were isolated from H-27 for proper control replicates. However, the limited results from the first two trials were in accord with those of the third (complete) one.

Table 6-4

Thymidine incorporation of Equine peripheral blood lymphocytes cultured with PHA for three days.

	CONTROL (c.p.m.)	PHA (c.p.m. \pm S.E.)	STIMULATION INDEX \pm S.E.
(H-27)L (H-27)S	420	9467 \pm 548	22.54 \pm 1.31
(H-27)L (G)S	345	12585 \pm 1304	36.48 \pm 3.78
(G)L (G)S	480	30417 \pm 1731	63.37 \pm 1.26
(G)L (H-27)S	415	31492 \pm 1958	75.88 \pm 4.72

G = control horse, L = lymphocytes, S = serum,
c.p.m. = counts per minute, and S.E. = standard error.

For both H-27 and the control horse there was a slightly higher stimulation with autologous serum. Species variation does occur, but the horse would seem to be one species in which lymphocyte stimulation by PHA is better when associated with autologous serum (P. Ramadass, pers. comm.).

H-27 presented a very slight buffy coat compared to other horses. This was noted both on the two inconclusive attempts and the one reported herein. In the last test the buffy coats from twelve 10 ml heparinized vacutainers of blood from H-27 were pooled for the Ficol separation, while only four tubes were used from the control horse. On the Ficol gradient no clear cloudy interface was present

in the H-27 samples, although the control horse samples had a slight one. The generalized cloudy area found in H-27's samples was extracted and subjected to an additional wash which did yield more than sufficient cells. Quantitative comparisons were not possible because one tube of 'normal' cells broke during the second wash. However, even with the loss of one half of the 'normal' cells sufficient numbers were obtained for the test. That is, they were obtained from the equivalent of one sixth the amount of whole blood as those from H-27.

The foregoing observations in no way reflect upon the test itself since equal numbers of live cells were used from both animals. However, it may have indicated a second condition in H-27, namely, relatively low numbers of circulating peripheral lymphocytes and/or a difference in their morphology. The species has been studied insufficiently so polymorphic variations on Ficoll gradients are not known (P. Ramadass, pers. comm.) and no blood counts or haemograms were done on H-27.

6.4. Discussion

There were two potentially significant aspects to H-27's case. First, the exact nature and extent of H-27's cytogenetic abnormality was not determined. That is, H-27 may have been a horse which demonstrated some of the possible limitations of in-vitro culture techniques in the accurate diagnosis of some mosaic/chimaeric conditions. She was confirmed, however, by sex-chromatin studies to have been mosaic/chimaeric for cells with either more than two X-chromosomes, polyploid nuclei or abnormal X-inactivation mosaicism. Secondly, the case raised questions of pleiotrophy and heterogeneity of defective lymphocyte function, cytogenetic abnormality and infertility. These questions suggested pathogenetic hypotheses of possible importance both to the cytogenetics of the horse and the application of cytogenetics to clinical evaluation in horses.

6.4.1. Cytogenetic Abnormality

The presence of a few neurons with two sex-chromatin bodies indicated that H-27 had some neurons with two inactive X-chromosomes. Since there had been no evidence, in any mammalian species, that cells without any active sex-chromosomes were viable, it was unlikely that these neurons were 64,XX. On the other hand, H-27's cultured cells had several hyperdiploid chromosome configurations. Some or one of these might have been present in the embryo and resulted in neuronal mosaic/chimaerism. Alternatively, a polyploid (4N) cell, of which there seemed to be relatively many in cell cultures, would also display two sex-chromatin bodies (Barr, 1966). Hyperploidy is known to be consistent, in the mosaic/chimaeric condition, with both viability and normal phenotype (Chu et al., 1964; Schindler and Mikamo, 1970; Dunn et al., 1970; Rieck, 1973). Therefore, it was considered probable that H-27 had a mosaic/chimaeric line (or lines) as an embryo which either persisted through adulthood or mosaic lines formed in-vivo due to mitotic instability.

Although mitotic instability in-vitro could effectively mask the presence of low level mosaic/chimaerism in-vivo (Benn, 1977), mitotic instability itself could be considered symptomatic of possible cytogenetic abnormalities. Ford and Lester (1978) found significant

differences in the proportions of abnormal (i.e., polyploid, hypodiploid and hyperdiploid) cells in cultures between phenotypically normal humans with minor chromosome alterations and control cultures. In addition, it has been suggested that some whole body chimaeras may be predisposed to mitotic instability (Stone et al., 1964; Fryns et al., 1978). In both these situations the indications were that the mitotic instability in-vitro reflected errors that occurred in-vivo. The large amount of cytogenetic information from cancer research also supported the reliability of in-vitro simulation of in-vivo situations and has demonstrated that cells with very abnormal karyotypes can exist and multiply in-vivo.

H-27's seeming mitotic instability itself may have been either a manifestation of a cytogenetic abnormality or have been a manifestation of a pathological condition. On the other hand, a combination of these factors might have occurred in H-27.

6.4.2. Some Implications of the Failure of Lymphocyte Cultures

H-27's lymphocytes consistently failed to grow in culture. Arai et al. (1977) proposed that the presence of certain serum proteins (albumin-bound fatty acids) was required for transformation of T-lymphocytes into dividing cells. A serum protein analysis, performed by the Department of Veterinary Clinical Pathology, showed that H-27's serum proteins were within normal limits. In view of this, a depressed lymphocyte proliferation response was implied.

Depressed lymphocyte proliferative response to PHA has been found in human patients with a variety of primary immune deficiencies, auto-immune diseases, virus infections, malnutrition, lymphomas and other malignancies, as well as in conjunction with many forms of therapy (Herberman, 1978). In addition, the in-vitro mitotic response had been shown to have an approximate correlation with the in-vivo situation and could be used to assess cellular immune competence (Hudson and Hay, 1976; Herberman, 1978).

The basis of lymphocyte culture is that in the presence of phytohaemagglutinin (PHA), T-lymphocytes transform into blast (dividing) cells. This can be referred to as lymphocyte transformation, blastogenesis, mitogenesis, proliferation or stimulation (Herberman, 1978). A depressed lymphocyte proliferative response reflects either a quantitative depression in reactive cells or an

intrinsic defect in the function of T-cells (Hudson and Hay, 1976; Herberman, 1978).

The difficulty encountered in isolating sufficient cells for the transformation tests from H-27's blood raised the possibility that she may have had fewer T-cells circulating than either a normal mare or stallion. This is one component of the lethal severe combined immunodeficiency syndrome of foals (Poppie and McGuire, 1977), so the existence of horses with decreased numbers of T-lymphocytes might be expected. The T-cells present in H-27, however, were shown to have been defective.

T-lymphocytes are primarily responsible for cell-mediated immunity, although they are believed also to be involved in most immune responses (Herbert, 1970; Herberman, 1978). Cell-mediated immunity is important in the resistance to and recovery from viral, fungal and intra-cellular bacterial infections. It is also important in tumour immunity and the rejection of aberrant tissue cells, resistance to larger parasites, delayed hypersensitivity reactions, and auto-immune disease (Herbert, 1970; Schalm *et al.*, 1975; Herberman, 1978). More than one major defect of leucocyte function in humans can lead to clinical syndromes. Not infrequently two or more signs of defective lymphocyte function are found in the same patient (Ryan and Majno, 1977). Some examples of defective lymphocyte function are: neutropenia, as in leukemias and cyclic neutropenia associated with the development of skin infections; disorders of migration and chemotaxis, as in intrinsic cellular dysfunctions such as diabetes mellitus and "lazy leucocyte syndrome"; and disorders of phagocytosis and microbial mechanisms (Ryan and Majno, 1977).

H-27's clinical picture, including a history of pyometra with which neutropenia is occasionally associated in mares (Hughes *et al.*, 1979), and the post-mortem findings did not provide evidence to disprove the hypothesis that defective T-cell function both existed in H-27 and influenced her phenotype.

6.4.3. Possible Implications of Mitotic Instability in Cell Cultures

All H-27's cell cultures seemed to have a high number of hyperploid and hyperdiploid cells. Hyperploidy and hyperdiploidy in cell cultures are associated with mitotic instability in-vitro.

All H-27's plasma clot cultures were set with homologous plasma. Moreover, some of the first skin cultures to grow out from the explants were started with growth media supplemented with non-heat inactivated serum. Therefore, the possibility of an auto-immune type reaction on the rate of hyperploidy in H-27's cell cultures could not be excluded. A significant increase in both hyperploidy and endoreduplicated mitoses in-vitro was found by Fialkow (1966) in allogenic cell culture systems, and chimaeric tetraploid cells arising from cell fusion following reciprocal cross sensitization has been reported (Fialkow, 1966).

Endoreduplication (Figure VI.9) has been proposed as involved in the etiology of some hyperploidy (Aspillaga et al., 1964). It is occasionally seen in human cell cultures (Schwaracher and Schnell, 1965) and is commonly found in some pathological conditions (Freedman et al., 1964; Lancet, 1964). The mechanisms causing the phenomena of endoreduplication are not known but are believed to involve failures of cellular structures or organelles (Houston, 1964; Kusyk and Hsu, 1979).

On the other hand, with the exception of Fryns et al.'s (1978) case, whose cells also showed an unusual degree of instability in culture, the only evidence for somatic cell recombinant "chimaeric" lines arising in-vivo comes from Haemopoietic chimaeras (Stone et al., 1964; 1968). In-vitro "chimaeric" cell cultures have been used to demonstrate aspects of cytogenetic instability, including somatic cell recombination via polyploid stages (Sprague and Martin, 1969). On the basis of current cytogenetic knowledge, therefore, it might be said that either the "chimaeric" condition predisposes to mitotic instability or mitotic instability predisposes to mosaic/chimaerism.

It was not possible to say that H-27's probable immune system defect was directly related to her cytogenetic anomalies or the reverse. However, since possible manifestations of defective leucocyte function, i.e., leukemias, diabetic conditions, auto-immune states, have been frequently associated in humans with chromosome abnormalities, a relationship between H-27's immune status and her cytogenetic presentation was a possibility.

6.4.4. Infertility

H-27 was an infertile mare. She had at least two potential components to her infertility. She was anatomically sub-fertile and there may have been a physiological incapacity to reproduce related to her cytogenetic picture.

Since H-27's right Fallopian tube ended blindly and lacked fimbriae,⁴ H-27 was structurally sterile with regard to any ovulation from her right ovary.

In the mare there is little difference between right and left ovarian activity (Arthur, 1958; Bain and Howey, 1975; Butterfield and Matthews, 1979). Histological evidence obtained post-mortem confirmed that both H-27's ovaries had been active. In the absence of any evidence to the contrary it must be assumed that one of the reasons for H-27's failure to conceive was this anatomical defect.

Infertility in a mare with an anatomical defect reducing her potential fertility does not necessarily imply there was a physiological abnormality as well. Any reduction in a mare's reproductive potential may of itself give the appearance of infertility under the restricted breeding practices of Thoroughbred management. Recurrent endometritis and consequent endometrial deterioration further complicated the evaluation of H-27's ability to reproduce.

H-27's endometritis might have been due either to a failure of the cellular immune component or the humoral components of the equine uterine protective mechanisms (Kenney and Khaleel, 1975). In either case, however, a relationship with defective T-cell function or numbers could be implied. The chronic nature of the endometritis, none-the-less, indicated H-27 had a predisposition to one form of infection highly correlated with infertility.

H-27 also had a potential for development of an auto-immune response related infertility. Her blind right Fallopian tube meant that the body's immune system was exposed to ova from the right ovary. Once the general immune system became sensitized, an auto-immune

⁴ It was interesting that paraovarian cysts, which are not uncommon in mares (Arthur, 1958; Smith et al., 1972), were present in H-27 only on the side where a (probably embryologic) mal-development of the mesonephric system had occurred.

response could have been triggered against ova from the left ovary within the (normally tolerant) reproductive tract. Even a minor auto-immune reaction could have upset the balance needed for normal fertilization, cleavage and ovum transport.

If one accepts the probability that H-27 had an immune system dysfunction, one must consider the possibility that this could have lead to formation of abnormal gametes, zygotes and embryos. The antigenic state of the ovum in some species is thought to be integral in allowing normal fertilization and cleavage (Scott and Jones, 1976). Altered immune responsiveness and auto-immune disturbance have been suggested as a cause of abnormal gametogenesis resulting in aneuploidy, polyploidy, and a high risk of spontaneous abortion (Fialkow, 1966; Fechheimer, 1968; Cassidy *et al.*, 1978).

The suggestion that H-27 may have been in foal at least once does not contradict the theory of immune system dysfunction having been involved in her infertility. A dysfunction could be compatible with conception while incompatible with some stage of pregnancy. Examples of such incompatibility are by affecting endocrine metabolic pathways, through association with diseases which create a hostile intra-uterine environment, and by affecting the delicate immune status required for the continuation of pregnancy (Beer and Billingham, 1976).

In addition, when there is a history of infertility mitotic instability in-vitro may also suggest the possibility of meiotic instability in-vivo (Ford and Lester, 1978), quite independently of the causes of that instability. Results from cell cultures from tissues of H-27 could be considered circumstantial but suggestive evidence that her cells were predisposed to mitotic, and therefore possibly meiotic, errors in-vivo.

6.4.5. Summary

H-27 was both cytogenetically abnormal and infertile. It was not possible to define the nature of her abnormality other than an apparent mitotic instability in-vitro and the presence in her neurons of some cells either with extra X-chromosomes or abnormal sex-chromosome activity. It was thought that H-27 could have been mosaic/chimaeric for a polyploid (4N) cell line. While such a line

could have been present embryologically, as shown by sex-chromatin studies, it was thought that H-27 was primarily a non-specific proliferative mosaic. That is, her cultured cells reflected an in-vivo mitotic instability. The probable in-vivo (and in-vitro) instability may have resulted from some other cytogenetic abnormality, such as whole body chimaerism or a structurally abnormal chromosome(s), or from some physiological or metabolic disturbance which itself may have arisen from a cytogenetic or genetic abnormality.

The information obtained from H-27 was insufficient to distinguish between the possible pleiotrophy and possible heterogeneity (Herrman and Opitz, 1977) of either her cytogenetic abnormality or her defective lymphocyte function and her infertility.

CHAPTER VII
GENERAL DISCUSSION

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CHAPTER VII

GENERAL DISCUSSION

7.1. Prevalence of the Mosaic/Chimaeric Condition in Domestic Horse Breeds.

The primary hypothesis of this study was that individuals with abnormal sex chromosome constitutions, the majority of which are mosaics or chimaeras, comprize a significant proportion of some E. c. caballus breeds, in particular the Thoroughbred. The relative ease with which the mosaic/chimaeric individuals in this Thesis were located was evidence in support of that hypothesis.

The current belief among human cytogeneticists is that mosaicism is more common than simple chromosome abnormalities. If this were true in domestic horses the prevalence of the XO mare alone suggested that a large number of mosaic/chimaeric horses would also be found in equine populations. This theory was supported when a 63,XO/64,XX filly was found during a pilot cytogenetic survey of 108 yearlings, only 60 of which were fillies (Walker and Bruère, 1979; Appendix I).

Although human cytogeneticists have established that sex chromosome mosaic/chimaeras can exhibit a range of phenotypes, it has often been phenotype deviation that has alerted them to the possibility of mosaic/chimaerism in humans. Horse breeders, on the other hand, usually enforce very rigid selection against horses that

have physical anomalies. Despite this, mosaic/chimaeric examples of all the major sex chromosome aneuploid conditions infrequently found in humans, XO, XXY, XYY and XXX, were found during cytogenetic analysis of less than 200 Thoroughbreds. The mosaic/chimaeras in this Thesis were from a concentrated, highly selected, population of "approximately 25,000 horses"¹, implying that the mosaic/chimaeric condition occurs frequently in the Thoroughbred.

Therefore, it was highly probable that the horses in this study were found because the mosaic/chimaeric condition is not unusual in the Thoroughbred.

H-47 (Chapter V) might have been considered an exception since she was examined for cytogenetic anomalies because of certain congenital defects. In this, her case resembles other reported cases of horses that had both phenotype deviations and an abnormal karyotype. However, it was only the combination of an excellent pedigree, an ethical owner/breeder devoted to his horses and the intervention of an enthusiastic veterinarian, which saved H-47's life and began the sequence of events that indirectly led to her cytogenetic analysis. She may have been either a rare case or an example of a mare that is seldom brought to the attention of researchers.

Of the remaining four cases in this study none were, as far as was known, particularly unusual for the breed. Full grown stallions and geldings of H-11's size (Chapter III) represent one extreme of the normal height distribution of the Thoroughbred. What distinguished H-11 was that his development toward that extreme was questioned by an educated owner and experienced veterinary surgeon. More research on foetal and neonate equine growth parameters must be done, and more information gathered on growth rates in horses with abnormal karyotypes, before it can be said that H-11's pattern of growth was unusual. Similarly, H-46's proposed masculine traits (Chapter V) may not have been unusual in a working, racing mare. There may even be a tendency for trainers to seek and develop such characteristics.

¹ No accurate survey of the Equine population of New Zealand exists. Registered Thoroughbreds at any given time do not include either foals and yearlings or Thoroughbreds on Pony and Hunt Registers. There is no information available on Thoroughbreds or animals of some Thoroughbred breeding which are not registered (H. Pearce, pers. comm.).

H-52 (Chapter IV), like the majority of animals reported in equine cytogenetic literature, was discovered to be cytogenetically abnormal during investigation of her infertility. Because of her small ovaries she had been suspected to be an XO mare. However, neither infertility, small ovaries nor the XO condition would seem to be very unusual in the domestic horse (Blue, 1976; Blue et al., 1978; Bruère et al., 1978; Trommershausen-Smith et al., 1979). H-52 was one of the few such animals investigated cytogenetically so that her real chromosome constitution was defined. H-27 (Chapter VI) was also infertile. However, it was the possibly atypical failure of her lymphocytes to grow in culture which suggested she might be cytogenetically abnormal, rather than her (at that time believed to be a acquired) infertility, which led to an intensive cytogenetic investigation. It is not known how unusual such a lymphocyte response is in horses. However, H-27's clinical condition, endometritis, is not uncommon in mares (Kennedy et al., 1978) and it may have been related to her cytogenetic anomalies.

7.2. Possible Predisposing Factors to the Mosaic/Chimaeric Condition and the Horses in this Study.

No overall pattern was found to suggest factors which might have predisposed to the formation of the mosaic/chimaeric horses presented in this Thesis. However, questions were raised which suggested areas where further study may be worthwhile.

7.2.1. Parental Age Effect

Trommershausen-Smith et al. (1979) reported no parental age effect in their XO (and presumably XO mosaic) cases, which had also been the experience of the Cytogenetics Unit at Massey University (Bruère, pers. comm.). This was in possible contrast to human cytogenetic theories (Emery, 1976; Warburton et al., 1980). However, the domestic horse, in particular the Thoroughbred, has a foreshortened reproductive life (Hutton and Meachem, 1968; Bain, 1969; Laing and Leech, 1975), which is subject to artificial selection pressures unrelated to reproductive performance.

The mean and range of parental ages at conception for the mosaic/chimaeric animals in this Thesis and XO mares for which pedi-

gree information was available are given in Table 7-1. The differences in parental ages were not significant. The slightly higher age distribution of the parents of XO mares towards the upper end of the Thoroughbred reproductive span may be non-biological. Fillies bred from horses which have raced were probably more likely to be assessed cytogenetically, and the majority of such stallions and mares do not begin stud duties until about the age of eight years.

Table 7-1.

Comparison of the ages at conception of parents of XO mares and the mosaic/chimaeras in this study.

	<u>AGE AT CONCEPTION</u>	
	<u>Mean</u>	<u>Range</u>
SIRES		
Mosaic/chimaeras (n = 6)	6.17	4 - 9
XO mares (n = 9)	8.11	4 - 12
DAMS		
Mosaic/chimaeras (n = 4)	6.75	5 - 10
XO mares (n = 10)	9.4	5 - 14

n = number of animals

Until demographic data is available on horse populations as a whole and chromosomally abnormal animals within these populations, the question of a biological significance in parental age on the frequency of chromosome abnormalities in mares and stallions cannot be answered.

7.2.2. Birth Order and Dam's Obstetric History

The sample was too small for there to have been any significance to the order of birth of the mosaic/chimaeras in this study; of the five mosaic/chimaeras studied, only H-27 was the first known conception of the dam. By comparison, two out of the ten dam's for which information was available had had an XO filly as the mare's first known conception.

None of the dams of the mosaic/chimaeras had an unusual obstetric history as far as could be discovered. All had rather better than average fertility, with the possible exception of H-11's dam, for which full information was not available. None of the dams were available for cytogenetic analysis.

7.2.3. Possible Influences of Metabolism and Physiology

Although much research has been done on the uniqueness of the reproductive cycle of the mare, no information was available on how aspects of this uniqueness may affect gametogenesis. Investigators have not agreed on the oocyte maturation stage when ovulation occurs in mares (Hamilton and Day, 1945; Roberts, 1971; Webel et al., 1977; Barone, 1979). It is possible that the hormonal condition in mares may influence oocyte maturation. The maturational stage of the oocyte at ovulation is one factor that can lead to dispermy and/or aberrant cleavage, which has been associated with the etiology of the mosaic/chimaeric condition.

In general the influence of metabolism and physiology on karyotype abnormalities, and the reverse, have not been studied in the horse. In humans significant correlation has been noted between the occurrence of specific diabetic conditions in patients with sex-chromatin positive Klinefelter's syndrome and monosomy-X and their families (Nielsen et al., 1969; Hsueh et al., 1979). Barnes et al. (1979) suggested that pregnancy itself could create a diabetic condition in some mares. The two XX/XXY mares in this study were both post partem conceptions. Since such conceptions are said to be at high risk of embryonic loss (DuPlessis, 1964; Merke, 1967; Dawson, 1977) and since embryonic loss is highly related to chromosome abnormalities, the possibility of a relationship between a diabetic condition and the incidence of chromosome anomalies in the horse might deserve investigation.

Irvine and Evans (1975) speculated on the incidence of clinical and sub-clinical hypothyroidism in the horse, a species which has a very high incidence of thyroid disease. The incidence of thyroid abnormalities is also high in Klinefelter men and maternal thyroid autoantibodies have been suggested to be involved with the etiology of Down's syndrome and monosomy-X (Fialkow, 1966, 1970; Engel et al., 1969; Mchardy-Young et al., 1970; Hseuh et al., 1979).

H-27's case (Chapter VI) reinforced the lack of knowledge of 'normal' as opposed to 'abnormal' cytogenetic variation in the horse. Her apparent mitotic instability could have reflected an abnormality of the immune system. On the other hand, the defect in T-cell function might have resulted from her cytogenetic abnormality. Since the immune system is intimately involved in normal development and fertility, cytogenetic studies of similar horses might prove of importance.

7.2.4. Inbreeding and Relationships between the Mosaic/Chimaeras

In agreement with Trommershausen-Smith (1979) no significance was found in the degree of inbreeding in the mosaic/chimaeras in this study. No significant difference was found between inbreeding coefficients of the mosaic/chimaeras and selected XO cases. However, inbreeding coefficients were not available for the Thoroughbred population of New Zealand as a whole and the comparison figures of Jones and Bogart (1971) and Cunningham (1975) may not have been applicable.

All the cases in this Thesis were related (Table 7-2). Two ancestors were common to all the mosaic/chimaeras and at least 15 horses appeared in the pedigrees of two or more of them. This was thought to reflect Thoroughbred breeding patterns, and to have no explicit significance in the frequency of the mosaic/chimaeric condition in Thoroughbreds.

Table 7-2

Approximate coefficient of relationship ($\times 10^{-2}$).

	<u>H-27</u>	<u>H-47</u>	<u>H-46</u>	<u>H-52</u>
H-11*	0.49	0.54	0.78	1.61
H-52	3.71	1.68	1.17	
H-46	1.95	4.59		
H-47	2.05			
*Sire pedigree only				

Only the paternal pedigree and part of the maternal grandsire's pedigree were available for H-11. Since H-11's dam was well bred it was considered likely that were a complete pedigree available for H-11 the relationship between him and the others would have increased. None the less, the relationship between H-11 and H-52 was not the lowest of the horses in this study and it was between two-thirds and twice as close as H-11's relationship to the other mares. H-46 and H-47, both XX/XXY mosaic/chimaeras, were the closest related of the horses in this study. However, H-27 was most closely related to all the mares. This was interesting because H-27 did not have a clearly defined mosaic/chimaeric condition. The relationships between her and the others, therefore, could have suggested a possible generalized influence on the presence of a mosaic/chimaeric condition.

No abnormality intrinsic to the X-chromosome was suggested. Among the four mares there was only a very slight possibility of a common ancestral X-chromosome and the only pattern that emerged was that some bloodlines were heavily represented in the population as a whole. Pedigree analysis did show that H-52, H-11 and H-46 shared a common ancestral Y-chromosome. However, no significance could be attributed to this in such a small sample. There was no evidence from pedigree studies to discredit the possibility of an autosomal (genetic) predisposition to chromosome anomalies.

It was interesting to note that the two non-intersexual XX/XXY mosaic/chimaeras (Chapter V) both of which were probably 'foal-heat' conceptions, by dams with good reproduction records, conceived to four year old sires, were also relatively closely related primarily through their sires.

7.2.5. Genetic Predisposition to Masked Karyotype Change

In the Thoroughbred it is probable that breeding practices may lead to a concentration of certain alleles. An example of gene concentration tending to chimaera formation was reported in the Hollaender strain of mice (Polani, 1973). H-11's case (Chapter III) suggested the possibility of an inherited predisposition to mitotic errors and meiotic errors which were carried by viable gametes. This was the only case where three generations had been investigated cytogenetically in the horse, therefore the uniqueness of this situation in horses was not known. However, H-11 was a mosaic/chimaera, was

unequivocally the sire of an XO filly, and there were suggestions of possible abnormalities in his sire, half-brother and another of his progeny.

The term "load" is used by geneticists to denote the extent to which a population departs from a perfect constitution. Many people believe that because humans have practised rigorous selection on the domestic horse, it can have only a very low either genetic or cytogenetic load (Kieffer, 1975). However, this may not be so when we consider the possibility in the horse of as yet undetected cytogenetic and genetic anomalies that have no visual manifestation, several of which have been discovered in humans. Even in this study, H-11, perhaps his sire, and H-27 could have been examples of horses that carried cytogenetic and/or genetic loads with no immediately obvious adverse effects. H-11 was fully fertile and H-27's infertility was, despite any predisposition to either infertility or infection, attributable to the development of endometritis.

It was suggested in Chapter III that the metaphase arrangements seen in H-11's sire, half-brother and one offspring (Figures III.8 and III.9) might represent either intra-chromosome rearrangements or fragility of the genome carried by these animals. Similar figures were seen in H-27's cultured cells (Figure VI.8). The same chromosomes were not involved in all these arrangements. H-27 was only distantly related to H-11, but on the other hand, her cultured cells displayed a far greater apparent mitotic instability in-vitro, which was one possible explanation for the irregularities in H-11's relatives and progeny. However, what was discussed in Chapter III concerning those metaphase chromosome arrangements was applicable to H-27, raising the question as to whether the possibility of some aspect of either the pleiotrophy or heterogeneity of cytogenetic abnormalities and H-27's phenotype (Chapter VI) might also have existed in H-11's kinship.

More fine structure analysis of E. c. caballus chromosomes is needed in regard to sub-chromosome polymorphisms, which at least in humans have been suggested to possibly lower reproductive fitness (Jacobs et al., 1975). For example, if either Nearco or Selene, through which a great many Thoroughbreds (including all the mosaic/chimaeras in this study) are related, had had a slight alteration in one chromosome, neither it nor its immediate descendants would

have necessarily suffered any selective disadvantage. Neither a slight tendency for chromosomally unstable offspring nor reduced fertility, which could have increasingly occurred as homozygous descendants entered the population, would necessarily be a disadvantage to a Thoroughbred. This would be especially the case in males which are usually gelded².

In addition there is no reason to suppose that karyotype changes in the horse have finished.

7.3. Mosaic/Chimaeric Individuals and Breed Sub-fertility

The secondary hypothesis of this study was that individuals with abnormal sex chromosome constitutions were, as a group, primarily responsible for the sub-fertility of the population. While no evidence was found during this study to cast doubt on this theory, the investigation of only five mosaic/chimaeric individuals could merely raise certain points of discussion.

7.3.1. Mosaic/Chimaerism and Fertility

The fertility of a population is influenced by both the numbers of sterile animals and the reproductive efficiency of the fertile members. The domestic horse may be particularly tolerant of the mosaic/chimaeric condition, as it is one of the animals under heavy selective pressure by humans for polygenic traits not associated with reproduction.

If the theory that human mosaics might be at risk of begetting offspring with abnormal chromosome constitutions (e.g., Barr et al., 1964; Morishima and Grumbach, 1968; Dewhurst, 1978) is correct, one could expect this to be fundamental throughout mammalian species.

H-11, an XY/XYY/?XO mosaic/chimaera was of proven fertility. Perhaps it was not just fortuitous that he sired an XO mare, H-68. Had that XO foal lived she would have been one more infertile mare lowering the fertility of the breed. However, there was also the suggestion that H-11's sire, half-brother and a colt by him, if not

²In the Family system of breeding the blood lines are carried through the dams, although it is progeny performance (primarily of their male offspring) that is evaluated.

mosaic/chimaeras might have been cytogenetically abnormal. This was the first time one of the relatively frequent XO mares was traceable to a mosaic/chimaera sire, and the first time a mosaic/chimaera had been found to belong to an equine family that may have had a cytogenetic anomaly.

The apparent frequency of XO mares, when considered in the light of human and equine cytogenetic literature, suggested that XO mosaicism would be the most frequently found type of mosaic/chimaerism. While an XO mosaic could be any extreme from fertile to sterile, a collection of XO mosaics would be expected to be less fertile than horses with normal karyotypes. This is because the simple XO form in woman has been, with rare exception, sterile, in the mare sterile and in the mouse sub-fertile.

However, assessment of negative aneuploid mosaicism in horses presents problems. While even one metaphase with an extra chromosome can be considered suggestive of possible mosaic/chimaerism, caution demands more stringent criteria be applied to metaphases lacking a chromosome. Furthermore, the basic cytogenetic work has not been done in Equidae to determine which chromosomes are preferentially lost through artifact and to determine the rates of age and sex related proliferative aneuploidy. In low-level mosaic/chimaeras errors are also bound to occur when artificial loss from an hyperdiploid cell gives rise to the appearance, in unbanded preparations, of either a normal 64,XY or 64,XX cell.

As Chapter VI demonstrated conservatism is necessary in diagnosing any mosaic/chimaeric condition in horses. Conservatism can lead to underdiagnosis of the mosaic/chimaeric condition. On the other hand, a number of reports of chromosome anomalies in horses suffer from lack of multiple cultures, inability or failure to culture multiple tissues and in some cases very few cells evaluated, such that the mosaic/chimaeric condition in individual animals may have been overestimated.

During this study one XO mosaic/chimaera yearling filly was confirmed (Walker and Bruère, 1979; Appendix I). This was a by-product of a subsidiary project aimed at determining the feasibility of cytogenetic surveys of horse populations for non-mosaic/chimaeric numerical chromosome abnormalities. During the process of establishing that the filly was an XO mare, it was confirmed as a mosaic/chimaera. One

infertile mare was tentatively diagnosed as an XO mosaic on the basis of multiple leucocyte cultures (Bruère et al., 1978; Appendix II). Several other horses were suspected of having cytogenetic anomalies including mosaic/chimaerism but were unavailable for sufficient study. H-11's (Chapter III) XO line could not be confirmed because banding was not done on chromosome preparations from his cultured cells.

Whether or not XO mosaic/chimaerism is prevalent in horse populations, the two mares with XXY mosaic/chimaerism, a stallion with XYY mosaic/chimaerism, and a mare with the complex mixoploidy of H-52 which included XXX cells and was probably based to a diploid number of 65, demonstrated that any kind of sex chromosome mosaic/chimaerism might be frequently found. H-27, an infertile mare further extended that to suggest that proliferative mosaicism, as well as perhaps polyploid mosaic/chimaerism, could also be found in horses.

If all kinds of mosaic/chimaeric sex chromosome combinations were to be found in physically normal horses with any regularity in the population, the XXY chromosome constitution, which in the simple form is sterile, could like the XO constitution be expected to decrease the population's fertility. However, there was little reason, based on human reports, to suspect the XYY and XXX configurations would bias a group's fertility distribution, unless the mosaic/chimaeric condition itself predisposed to either conceptual loss or production of sterile offspring.

Of the few mosaic/chimaeric animals examined in the literature, most, for example the two XO/XX mosaics of Hughes et al. (1975, 1977) and in this Thesis H-52 and H-47, have shown a possibility of the capacity to reproduce. They either had slight oestrous behavior and ovarian activity or known conception. Furthermore, of the four mares in this study, only H-27 was in all probability functionally infertile. Had hormone therapy been available H-52 and possibly H-46, like H-47, might have at least conceived. Any of these mares, with the exception of H-27, might have been termed "shy breeders".

As more "shy breeders" are induced through artificial synchronization of oestrus, more mares which may be chromosomally abnormal may be served. These mares could be at high risk of either conceptual loss or producing offspring with abnormal chromosome constitutions. In either event, the fertility of the breed would decline.

7.4. Cytogenetics and the Domestic Horse

The cases in this study presented three aspects of the field of cytogenetics applied to the domestic horse. The first aspect was the identification, investigation and definition of individuals with cytogenetic abnormalities. This is fundamental to cytogenetics and was involved in all the cases presented. Further to this, in Chapter III and to a lesser extent Chapter VII, interrelationships between cytogenetics and genetics were displayed and discussed; thus indicating that in the horse, where pedigree information is often well documented, there is considerable scope for additional research of this nature. Finally, in Chapter VI, there was a tentative demonstration of the value of medical cytogenetics applied to a mare with a difficult clinical problem. This was an excursion into the relatively unexplored potential of cytogenetics as a part of comprehensive veterinary health investigations.

In summary this study has revealed some of the potential the cytogenetic discipline has in developing our understanding of both infertility and fertility in the domestic horse. Clearly this potential is not restricted to the equine species, but rather could be extended to include all domestic animals.

APPENDICES

APPENDIX I

1978 PILOT SURVEY

Introduction

A pilot cytogenetic survey of Thoroughbred yearlings was done in August, 1978. The aim of the survey was to test the feasibility of large scale studies of this kind in the domestic horse. However, it was hoped that the results of the survey would also provide information on the frequency of horses with abnormal chromosome counts, in the simple non-mosaic and non-chimaeric condition.

Materials and Methods

In August, 1978, blood samples were obtained from 112 yearlings, 49 colts and 63 fillies. The yearlings were from eleven properties in the North Island of New Zealand, and were by at least 43 stallions (one property would not provide the names of sires). Cytogenetic screening was done on 108 of those yearlings, 48 colts and 60 fillies.

For animals 801 through 859 at the time the sample was obtained the yearling's sire, dam, and phenotypic sex (as reported by the owner) was recorded in a master book next to a preprinted number. This number was recorded on the blood sample tube. For animals 860 through 8113, the dam's name and the yearling's sex was put on the blood sample tube and a separate sheet was provided by the veterinarians giving the dam, sire, yearling's sex and the property where obtained. When these samples arrived at the laboratory, the information was transferred to the master book and the number was placed on the blood sample tube.

One tube of heparinized blood was obtained from each yearling. Blood samples 801 through 844 and 860 through 865 were collected on August 19, 1978. These were packed in ice within 15 minutes of

collection, refrigerated overnight at 4°C and were repacked in ice for transport to the laboratory. Blood samples 845 through 859 were obtained on August 20, 1978, and were packed in ice within 15 minutes of collection. Samples 866 through 8113 were obtained on the 23rd and 24th of August, 1978. Those obtained on the 23rd were refrigerated overnight and both lots were packed in ice, in an insulated container, and were flown to Palmerston North.

Cultures 801 through 865 were made August 20, 1978, and cultures 866 through 8113 on August 24, 1978. The procedures used are described in Appendix VI.

Two culture tubes were set-up from each blood sample. For both lots, the two culture tube sets were kept separate. Individual tubes from each set were randomly divided into groups of ten (centrifuge capacity). Colchicine was added to the groups at ten minute intervals, and harvesting occurred in the same, staggered, sequence three hours later. Fixed cells were refrigerated overnight. One slide was then made from each culture tube and a third slide from the cells of the two culture tubes combined.

A minimum of four cells were counted initially. The criteria was that chromosomes be countable. This was later upgraded to a minimum of four cells of which one could either be counted differentially, that is acrocentrics and non-acrocentrics could be identified, or it could have been karyotyped.

Chromosome counts were recorded against the yearling's number in the culture book. Every ten or so animals, the results were checked against the master book. When a discrepancy was noted, a request was made to the relevant veterinarian for a second blood sample. The veterinarian was not advised as to the reason for the request and at least one other second sample was requested from the same property. Repeat cultures were performed on seven yearlings in October, 1978, and on four yearlings in November, 1978.

Results

Four cultures (3.6%) failed to reach the minimum criteria for inclusion in the survey. A 96% success rate, however, was considered good for a survey of this size. One 64,XX filly was transferred from the survey, but was included in calculating the success rate. Chromosome counts from the pilot survey are provided in Table A-I-1.

Table A-I-1

The distributions of chromosome counts in the 1978 pilot survey.

FOAL NO.	SEX	COUNT DISTRIBUTION					P	TOTAL CELLS	FOAL NO.	SEX	COUNT DISTRIBUTION					P	TOTAL CELLS
		≤62	63	64	65	>65					≤62	63	64	65	>65		
801	M	-	-	5	-	-	-	5	860	M	-	-	4	-	-	-	4
802	M	-	-	5	-	-	-	5	861	M	-	-	4	-	-	-	4
803	M	-	-	4	-	-	-	4	862	M	-	-	5	-	-	-	5
804	F	-	-	5	-	-	-	5	863	F	-	-	4	1	-	-	5
805	F	-	-	4	-	-	-	4	864	F	-	-	6	-	-	-	6
806	F	-	-	5	-	-	-	5	865	F	-	-	4	1	-	-	5
807	M	-	-	4	-	-	-	4	866	F	1	-	4	-	-	-	5
808	M	-	-	4	-	-	-	4	867	F	-	-	3	-	-	1	4
809	M	-	-	5	-	-	-	5	868	F	-	-	6	-	-	-	6
810	M	-	-	5	-	-	-	5	869	F	-	-	3	1	-	-	4
811	M	-	-	5	-	-	-	5	870	F	-	-	4	-	-	-	4
812	M	-	-	4	-	-	-	4	871	F	-	-	5	-	-	-	5
813	F	-	-	5	1	-	-	6	872	F	1	-	4	-	-	-	5
814	F	-	-	6	-	-	-	6	873	F	-	-	4	1	-	-	5
815	F	-	1	4	-	-	-	5	874	F	-	-	4	-	-	1	5
816	F	-	-	5	-	-	-	5	875	F	-	-	4	-	-	-	4
817	M	-	-	5	-	-	-	5	(876)	(F)	-	-	-	-	-	-	-
818	M	-	-	4	1	-	-	5	877	F	-	1	3	-	-	-	4
819	M	-	-	4	-	-	-	4	(878)	(F)	-	-	-	-	-	-	-
820	F	-	-	4	-	-	1	5	879	F	-	2	3	-	-	1	6
821	F	1	-	4	-	-	-	5	880	M	-	-	4	-	-	-	4
822	F	-	1	4	-	-	-	5	881	M	1	-	3	-	-	-	4
823	F	-	-	4	-	-	-	4	882	M	1	-	4	-	-	-	5
824	F	-	-	4	-	-	-	4	883	M	-	-	4	-	-	-	4
825	F	-	2	3	-	-	-	5	884	M	-	-	4	-	-	-	4
826	F	-	-	5	-	-	-	5	885	F	-	1	6	-	-	-	7
827	F	-	-	4	-	-	-	4	(886)	(F)	-	-	-	-	-	-	-
828	F	-	-	4	-	-	-	4	887	F	-	-	4	-	-	-	4
829	M	-	-	4	-	-	-	4	888	F	1	-	6	-	-	-	7
830	F	-	-	4	-	-	-	4	889	F	-	-	5	-	-	-	5
831	F	-	-	5	-	-	-	5	890	F	-	-	4	-	-	-	4
832	M	-	-	4	-	-	-	4	891	M	-	-	4	-	-	-	4
833	M	-	-	4	-	-	-	4	892	M	-	-	4	-	-	-	4
834	F	-	-	4	1	-	-	5	893	M	-	1	3	-	-	-	4
835	M	-	-	5	-	-	-	5	894	M	-	-	5	-	-	-	5
836	M	-	-	4	-	-	-	4	895	M	-	-	4	-	-	-	4
837	M	-	1	3	-	-	-	4	(896)	(M)	-	-	-	-	-	-	-
838	F	1	-	5	-	-	-	6	897	M	-	-	4	-	-	-	4
839	F	-	1	5	-	-	-	6	898	M	-	-	5	-	-	-	5
840	M	-	-	4	-	-	-	4	899	M	-	-	9	-	-	-	9
841	F	-	-	5	-	-	-	5	8100	M	-	1	4	-	-	-	4
842	F	-	-	4	-	-	-	4	8101	M	-	1	4	-	-	-	5
843	M	-	2	4	-	-	-	6	8102	F	1	22	3	-	-	-	26*
844	M	-	-	5	-	-	-	5	8103	F	-	-	4	-	-	-	4
845	F	-	-	5	-	-	-	5	8104	F	-	1	3	-	-	-	4
846	M	-	-	4	-	-	-	4	8105	F	-	-	4	-	-	-	4
847	M	-	-	5	1	-	-	6	8106	F	-	-	4	-	-	-	4
848	M	-	-	4	-	-	-	4	8107	F	-	-	4	-	-	-	4
849	M	-	-	5	-	-	-	5	8108	F	-	-	4	-	-	-	4
850	M	-	3	11	-	-	-	14	8109	F	-	-	6	-	-	-	6
851	M	-	-	4	-	-	-	4	8110	M	-	-	4	-	-	1	5
852	M	-	-	4	-	-	-	4	8111	F	-	-	4	-	-	-	4
853	F	-	-	6	-	-	-	6	8112	F	-	-	4	-	-	-	4
854	F	-	-	5	-	-	-	5	8113	F	-	-	5	-	-	-	5
856	F	-	3	5	1	-	-	9									
857	F	-	-	4	-	-	-	4									
858	F	-	2	3	-	-	-	5									
859	Deleted from survey																
								Total			8	46	469	9	0	5	537

P = polyploid cells
M = colt
F = filly

* These were from the first leucocyte culture. An additional 18 cells were evaluated from a second leucocyte culture.

One filly, H8102, was tentatively diagnosed as 63,XO. Additional cells were counted which suggested XO/XX mosaicism. A second leucocyte culture confirmed that she was a 63,XO/64,XX mare. A total of 44 cells were evaluated from this filly. Twenty-nine (65.9%) had 63 chromosomes, all of which karyotyped were 63,XO. In addition, 12 metaphases (27.3%) were 64,XX.

Three yearlings had been erroneously recorded, H8042 and H8065 as female and H8043 as male. In each case, the report of the yearling's phenotypic sex accompanying the second blood sample corresponded to its cytogenetic sex as determined by the first leucocyte culture.

The cultures from seven yearlings raised questions as to possible cytogenetic abnormalities, i.e., mosaicism and chimaerism. However, it was outside the scope of the project to investigate these.

Discussion

The pilot survey was designed for the detection of simple (non-mosaic and non-chimaeric) numerical chromosome anomalies. At the time of the survey no large scale cytogenetic survey of the domestic horse had been reported, so it was based upon early human population studies and those where resources have been limited.

A relatively unselected equine population was desired. However, selection had already occurred within the yearlings. The subjects were 3/4 year old, so foals with neonatal and early development problems had already been eliminated by the breeders and owners. The willingness, and conversely unwillingness, of some breeders and owners to cooperate was a factor in selection. However, a range of size and types of horse management establishments was included, which might have countered any possible bias in that regard. Purely temporal selection also was encountered. The 19th of August was cold, wet and windy, and a major sporting event was televised that day.

In addition, anxious and excited animals were not pursued, out of both concern for injury, and the belief that excitement in horses seriously jeopardizes the chance of successful leucocyte culture (Blue, 1976). The behaviour of some XO mares has been reported to be 'difficult' (Blue, 1976). Increased anxiety is a feature of the human XYY genotype and some XXY men (Hunter, 1969, 1977; Hook, 1979) as well as some other people with chromosome anomalies. Should this

be the same in domestic animals, unbroken horses with chromosome abnormalities would not be readily caught in the paddock.

Had the survey been conducted earlier in the year the possible effects of both anxiety on the part of the yearlings and the lack of foals with early development abnormalities might have been lessened.

The discovery of H8102, an 63,X0/64,XX filly, raised the possibility that chromosomally abnormal foals could occur with exceptionally high frequency. This X0 mosaic/chimaera was found among only 108 yearlings, 60 fillies, screened for cytogenetic abnormalities. By comparison either 500 infants could have been screened before one with any sex chromosome anomaly was found, or 2500 infants before an X0 was located (Boué and Boué, 1974).

The X0/XX mosaic/chimaera presented no signs of phenotype abnormalities. H8102 was not available for further blood samples or additional tissues for cell cultures. However, her owner considered her one of his best yearlings. Her size, Thoroughbred conformation and temperament were normal in the opinion of both the owner and veterinarian. As a two year old, H8102 might have been slightly large for her age, although normal size for progeny of her sire, according to her owner. He also reported that she was in all other ways normal and a good example of the breed. As the mare was to be raced, her reproductive potential was not yet a consideration and information on the development of her reproductive tract was not available.

The pilot survey was designed for detection of simple numerical chromosome anomalies. Hassold *et al.* (1978), upon whose work this survey was partially patterned, evaluated only four cells and were specifically not seeking mosaics. They did not discover any, and it was assumed that mosaic/chimaeras would not be detected in the pilot survey. This, in fact, was so, since within the limits of the pilot survey an X0 filly was found. Only when more metaphases were evaluated was H8102's 64,XX cell line discovered.

The pilot survey was designed to have only one stage. However, the discovery of a potential X0 mare, confirmed as a mosaic/chimaera in an extended inquiry, was in keeping with the literature.

The majority of human cytogenetic population surveys are designed in more than one stage. In the six studies reviewed by Hook and Hamerton (1977), the group that analysed the highest number

of cells in Stage I was the smallest survey and they detected no mosaicism. On the other hand, the group that analysed the largest amount of samples (13,939) only analysed a total of ten cells, two cells for Stage I, three for Stage II and five for stage III (Hook and Hamerton, 1977). That group discovered seven mosaic individuals. Moreover, according to Maeda et al. (1978), a European group found the abnormal cell line in 40, out of their 44 mosaic cases, among the first five cells evaluated.

The success of the pilot survey confirmed the feasibility of cytogenetic population studies of the domestic horse, when these are limited to simple numerical abnormalities. Further stages would, be necessary, however, to screen for mosaic/chimaerism. Difficulties were encountered in follow-up on pilot survey animals. These difficulties are unlikely to lessen until mosaic/chimaerism can be defined in terms of either breeding potential or the horse's performance. However, the discovery of one mosaic/chimaeric yearling in the survey argued against the idea that the karyotype of the horse was too complex for the application of human population survey techniques.

The discovery of one yearling with an abnormal karyotype also suggested that these animals may be relatively frequent in the population.

CORRESPONDENCE

XO CONDITION IN MARES

Sir, — In August 1978, we conducted a pilot cytogenetic survey to screen for non-mosaic sex chromosome abnormalities in a horse population. Prior to this survey eight XO mares had been examined by this laboratory in connection with infertility^(1,2). We have since verified four additional cases. The XO condition in the mare is characterized by either absent, or highly irregular, oestrous cycles, flaccid cervix, small uterus and very small ovaries without follicles. All XO mares so far examined appear normal but, in some cases, may be smaller in stature than other horses of the same breed.

The XO condition in mammals has been studied extensively in the mouse and the human, and reported in the cat and pig. In the mouse, monosomy X is associated with reduced reproductive life and reduced fertility (due to failure of the embryo to implant)⁽³⁾. Turner's Syndrome in humans is characterized by small stature, infantile external genitalia, streak gonads, primary amenorrhoea, webbed neck and, often, various clinical abnormalities such as skeletal, renal, cardiac and cortical anomalies.

In humans, sex chromosome mosaicism, though rarely found in abortion studies⁽⁴⁾, is more frequent than the simple extra, or missing, sex-chromosome condition. Mosaicism is not necessarily associated either with infertility, reduced fertility, or clinical abnormalities. Thus, the XO mare in some ways resembles both the XO mouse and one extreme of the human XX/XO mosaic (for example, they appear normal externally); while in other ways, such as being infertile, the XO mare resembles the Turner's human. We are currently investigating the relationship of mosaicism to infertility in the mare.

It should be emphasized that 10% of all human spontaneous abortions, 25% of those with chromosome abnormalities, are reported to be XO⁽⁴⁾. However, Turner's Syndrome is the least frequent sex chromosome anomaly in newborn infants: 0.4/1000, compared with XXX at 1.2/1000, XXY (Klinefelter's Syndrome) at 2.1/1000 and XYY at 1.3/1000⁽⁵⁾. The low numbers of XO's in the live-born human population is because less than 5% of such conceptuses go to term⁽²⁾.

Assuming equal viability for embryos with sex chromosome abnormalities, there should be similar proportions of XXX, XXY and XO horses in the population. In any case, it should be possible to find cytological evidence of XXX, XXY and XYY horses, unless these conditions are non-viable in this species. In humans, the XXX condition is seldom associated with infertility or phenotypic abnormalities, and appears more frequently than the XO condition in the live-born population. Likewise, the XYY male, though often appearing larger than average in stature, is both fertile and phenotypically normal. The XXY condition, Klinefelter's Syndrome, on the other hand, is associated with eunuchoid proportions, small testes and smaller than average penile dimensions, and in some cases low mentality and moderate-to-extreme feminization, i.e., gynecomasty, body-hair distribution and structural conformation. However, some XXY's appear quite normal and have been discovered only through infertility studies. So far, we have found XYY and XXY cell lines in phenotypically normal horses, but only in the mosaic state. The simple XXX, XYY or XXY condition may be present, but unnoticeable, in the horse population. On the other hand, these chromosome conditions may be selected against as embryos or neonates.

The apparent surplus of XO mares, however, indicated that some investigation should be made to ascertain if such a condition, and, perhaps, other sex-chromosomally abnormal but

phenotypically normal animals, were a significant part of the horse population.

In co-operation with H. Dewes and J. J. Hope, veterinarians, and several interested members of the N.Z. Thoroughbred Breeders' Association, 112 randomly selected yearlings from 11 studs in the North Island were bled, and the samples cultured using standard whole-blood techniques. Results of the chromosome complement were obtained from 108 samples: 48 colts and 60 fillies. One XO was found in the 60 fillies, and was subsequently confirmed as an XO/XX mosaic. No abnormal cell lines were found among the 48 colts, within the limits of the survey. However, the number of cells counted per animal (with the exception of the XO) were too few to eliminate the reasonable possibility of mosaicism.

The fact that, in this limited survey of 60 fillies, one XO mosaic occurred, together with our previous results, suggests that both the XO condition and XO mosaic conditions are possibly more prevalent in the mare than is recognised. We plan to extend the pilot survey, in the belief that serious consideration and research is needed to determine whether not only the XO condition, but any other sex chromosome abnormality, is a significant part of the poor reproductive performance of the thoroughbred.

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14th December 1978

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APPENDIX II

Table A-II-1 lists previously unpublished case summaries of seven XO mares diagnosed by the Cytogenetics Unit, Department of Veterinary Clinical Sciences, Massey University.

In addition, seven animals investigated for chromosome abnormalities, for which there was insufficient information to either confirm or refute the suggestion of a possible anomaly are provided in Table A-II-2.

Table A-II-1

Summary of unpublished cases of XO mares in New Zealand.

HORSE	BREED	AGE (years)	HISTORY	TISSUE	CHROMOSOME COUNTS					Total*	COMMENTS
					≤ 62	63	64	65	>65		
H-25	TB	11	"Shy breeder"	leucocyte	7	8	45	1	-	61	Some metaphases had two unpaired meta- or sub-metacentric chromosomes, suggesting the possibility of mosaicism for a structurally abnormal X-chromosome. Only 4 metaphases were 63,XO.
H-29	TB	-	"Shy breeder"	leucocyte	10	4	51	2	-	67	Both metaphases with 65 chromosomes contained an extra acrocentric, possibly a Y-chromosome.
H-30	TB	3	Infertile	leucocyte	13	22	75	4	11	125	All metaphases with more than 65 chromosomes were polyploid. The four cells with 65 chromosomes had different karyotypes. In addition there were many cells containing abnormal chromosomes which were not evaluated.
H-33	SB	6	"Shy breeder" got in foal with hormone therapy.	leucocyte	3	4	15	2	-	24	The possibility of mosaic/chimaerism could not be excluded on the basis of one leucocyte culture.
H-44	½-TB	6	Infertile with small internal genitalia and gonads..	leucocyte	4	9	23	6	-	40	Only three metaphases with 63 chromosomes were XO, however the six with 65 chromosomes all contained an extra sub-metacentric chromosome. These karyotyped as Triplo-X cells.
H-19	TB	8	Infertile. This mare was reported in Bruère <u>et al</u> 1978 and was an XX/XO mosaic.	leucocyte	24	38	86	1	-	149	A few metaphases with 64 chromosomes had two unpaired non-acrocentric chromosomes, of which one was the X.

TB = Thoroughbred, SB = Standard Breed

*Total = Total cells evaluated.

Table A-II-2

Summary of cytogenetic investigations of horses which had a suggestion of an anomaly, but for which there was insufficient information to verify.

HORSE	BREED	AGE (years)	HISTORY	TISSUE	CHROMOSOME COUNTS					Total*	COMMENTS
					62	63	64	65	>65		
H-25	TB	11	"Shy breeder"	leucocyte	7	8	45	1	-	61	Some metaphases had two unpaired meta- or sub-metacentric chromosomes, suggesting the possibility of mosaicism for a structurally abnormal X-chromosome. Only 4 metaphases were 63,XO.
H-29	TB	-	"Shy breeder"	leucocyte	10	4	51	2	-	67	Both metaphases with 65 chromosomes contained an extra acrocentric, possibly a Y-chromosome.
H-30	TB	3	Infertile	leucocyte	13	22	75	4	11	125	All metaphases with more than 65 chromosomes were polyploid. The four cells with 65 chromosomes had different karyotypes. In addition there were many cells containing abnormal chromosomes which were not evaluated.
H-33	SB	6	"Shy breeder" got in foal with hormone therapy.	leucocyte	3	4	15	2	-	24	The possibility of mosaic/chimaerism could not be excluded on the basis of one leucocyte culture.
H-44	½-TB	6	Infertile with small internal genitalia and gonads.	leucocyte	4	9	23	6	-	40	Only three metaphases with 63 chromosomes were XO, however the six with 65 chromosomes all contained an extra sub-metacentric chromosome. These karyotyped as Triplo-X cells.
H-19	TB	8	Infertile. This mare was reported in Bruère <u>et al.</u> 1978 and was an XX/XO mosaic.	leucocyte	24	38	86	1	-	149	A few metaphases with 64 chromosomes had two unpaired non-acrocentric chromosomes, of which one was the X.

TB = Thoroughbred, SB = Standard Breed

*Total = Total cells evaluated.

APPENDIX III

ANCILLARY INFORMATION TO CHAPTER IVA. H-52's Dimensions

Height at withers:	151.25 cm	(15.½ h.h.)
Croup Height:	152.50 cm	
Girth:	182.00 cm	
Body Length:		
shoulder to rump	167.50 cm	
shoulder to hip bone	117.50 cm	
total (straight line)	202.50 cm	
Head Length:		
top of head to a line between upper margin of nostril	47.5 cm	
inner corner of eye to upper margin of nostril	25.0 cm	
Legs:		
length from point of elbow to ground (leg vertical)	47.5 cm front 57.5 cm rear	
length from point of hock to ground (shank vertical)	15.0 cm front 12.5 cm rear	
circumference below the knee	20.0 cm front 22.5 cm rear	
length hock to fetlock	45.0 cm rear	
Weight:	431.82 kilograms	

B. H-52 POST MORTEM

The post-mortem on H-52 was conducted on 17th September, 1979, by Dr. A. Johnstone, Department of Veterinary Pathology, Massey University. Prof. D. Fielden and Mr. M. Birtles consulted on the reproductive tract and histology respectively.

The mare was not in oestrus and was not pregnant. Her condition was poor, however all organs and tissues were essentially normal.

- (1) A firm adhesion, 8 x 4 cm, was located between the spleen and left abdominal wall.
- (2) There were chronic fibrous adhesions between the liver and diaphragm.
- (3) Parasitic aneurisms were present with larve of Delafondia vulgaris
- (4) Chronic ulcerative gastritis, associated with Gastrophilus nasalis in the margo plicata and pylorus, was present in the stomach. There was mild hyperplasia of the epithelium.
- (5) The lungs were grossly normal, with non-suppurative chronic bronchitis and chronic focal pleurisy indicated histologically.
- (6) The heart was normal, but had multiple focal parasitic scars.
- (7) The spleen was congested. Histologically there were signs of chronic passive congestion and mild haemosiderosis.
- (8) The intestines were congested.
- (9) The pituitary was normal. No lesions were present histologically however a small thickened collagenous mass was present.
- (10) The pancreas was normal, but it had excess fibrous tissue around ducts.
- (11) There were mild centrilobular changes in the liver due to the presence of fine to course cytoplasmic vacuolation(i.e., fat).
- (12) The adrenal gland appeared normal. The normal distribution of neurons was present in the medulla and there were no microscopic lesions. There was focal vacuolation (probably fat) in the zona fascicularis and glomerulosa. Accessory islands of cortical tissue were present adjacent to the right ovary.

- (13) There was fibro-muscular thickening of the media and externa of the cranial mesenteric artery. There were also areas of acute and chronic inflammatory change with chondroid and myxomatous metaplasia, along with dystrophic calcification and verminous endarteritis (aneurism).
- (14) The branch of the internal carotid was normal.
- (15) The kidney was macroscopically normal. Histologically there was evidence of focal inflammation, focal calcification of tubules and mesangial thickening indicating mild glomular nephritis and mild chronic focal interstitial nephritis.
- (16) The thyroid was normal.
- (17) The brain and central nervous system were normal.
- (18) The left ovary measured 4 x 2 x 2.5 cm. A 2 mm diameter section of orange luteal tissue with a central brown area was present macroscopically, as were one or two brown haemorrhagic areas. Histologically, only stroma was seen, with no evidence of follicles. However, there were areas of local haemorrhage, between 14 days and several months old, which were both Pearl's Iron and Sudan Black positive.
- (19) The right ovary measured 3.5 x 2.5 x 1.5 cm. It contained brown haemorrhagic spots and a paraovarian cyst was adjacent to the ovary in the mesovarium. Histologically there was no evidence of follicles and only stroma was seen. There were areas of scarring with a centrally big area of haemosiderin and some cells filled with light brown granular material, which were both Pearl's Iron and Sudan Black positive.
- (20) The uterus measured 15 cm from horn to horn, and 12 cm from the internal os to the vulvar cleft. Both Fallopian tubes were patent and were normal histologically.
- (21) The left uterine horn had fairly low columnar epithelium and some dilated ducts containing proteinaceous material. The cytoplasm of the columnar cells was foamy. There were some nests of lymphoid cells, indicating some focal inflammatory change, and slight atrophy.
- (22) The right uterine horn was similar to the left one except the surface epithelium appeared almost cuboidal.

- (23) The mucosa layer of the upper uterine body had gland ducts developed, though relatively less dense than the lower region, which were tortuous but had fewer branches than those in the lower region. The epithelial cells were less columnar than in the lower region.
- (24) In the lower uterine body there were glands and groups of glands in the stratum compactum. The cells were low columnar with basal nuclei and rather foamy cytoplasm. Some contained a proteinaceous secretion. The surface epithelium was low columnar type with deeply staining cytoplasm.
- (25) The cervix had a single layer cuboidal lining, and there was slight mucus production.
- (26) The vagina was lined by stratified squamous, non-keratinizing, epithelium with numerous small inclusion cysts, which contained fibrillar clumps of mucus and cellular debris. Beneath the stratum germinativum and in places penetrating into lower levels of the epithelium were follicles of actively proliferating lymphoid tissue. In the lower vagina there were a few small chronic inflammatory nodules under the stratum germinativum. However, there were no inclusions and the epithelium was not as high as in the upper regions. The muscle layers appeared to be normal throughout the vagina.

C. ESTIMATES OF PRIMORDIAL CELL POOL SIZE

For the estimation of primordial cell pool size, the estimated proportions of cell types (i.e., XXX, XXY) in each solid tissue studied were considered a replicate estimation of the total mosaic/chimaerism present in the mare. The estimation of the primordial cell pool, therefore, probably became an estimate of either the undifferentiated pre-blastocyst or embryonic mass of the blastocyst.

The variance of a binomial distribution (Variance or $s^2 = pq/N$) was used for calculation of N, where N was the estimate of the primordial pool size that could have given rise to the mixoploidy estimated. The overall frequency with which a cell type was found in non-lymphocyte cultured cells was "p" and "q" was equal to 1 - p. It was outside the scope of this Thesis to evaluate the validity of the assumptions necessary for such a calculation (see Nesbitt, 1971; Nesbitt and Gartler, 1971; Fialkow, 1973; Falconer and Avery, 1978).

Table A-III-1 gives the estimated primordial cell pool sizes and 95% confidence intervals for the variance (estimated) in H-52 for 65,XXX, 65,XXY and adjusted 64,XX cells.

Table A-III-1

Cell Type	N (pool size)	95% confidence interval of N
XXX (p = 0.9103)	2.51	1.2 - 4.28
XXY (p = 0.4598)	7.51	3.4 - 12.2
XX (p = 0.0437)	2.24	1.1 - 3.8

A similar calculation performed for 66,XXXX and 66,XXXY cells yielded large pool size estimates, 17.5 and 27.2 cells respectively. Large pool size estimates could be expected if a cell line were at either a proliferative or selective disadvantage in-vivo. However, aside from introducing a large bias into the calculation, when analysis is performed on cell lines having a low estimated frequency it

becomes indefinite exactly which primordial pool one is estimating (Fialkow, 1973). In the cases of the adjusted XX cells, the XXXX and XXXY cells, one may be merely estimating the precursor pool size of those tissues exhibiting these lines.

APPENDIX IV

H-27, POST MORTEM

The post mortem, Number 11851, was conducted on 5th June, 1979, by Dr. Maurice Alley, Department of Veterinary Pathology, Massey University. Histological evaluation was also performed by Dr. Alley.

- (1) Irregular scurfy lesions, up to 5 cm diameter, were located over the entire body, but especially on the flanks and lower limbs. These were areas of hyperkeratosis and epithelial erosion, with replacement by a fibrinous and cellular exudate containing bacteria (cocci). However, no fungal elements were seen, and no dermatophilus was isolated from the skin.
- (2) A 3 cm diameter firm nodule was present on the bulb of the right hind limb. It consisted of proliferating immature fibrous tissue forming whorls in the upper dermis covered by acanthotic epithelium.
- (3) A well differentiated papilloma was present on the third eyelid of the left eye, which measured 1 cm x 4 cm.
- (4) The hind limbs were swollen up to the inguinal area with subcutaneous oedema of pale yellow colour. No reason for this oedema was found.
- (5) The lung showed moderate peripheral alveolar emphysema and bronchial smooth muscle hypertrophy.
- (6) The right Fallopian tube was enlarged (5 mm in diameter). It had no fimbrii and ended "blindly" at the right ovary. Five fluid filled cysts (5 mm in diameter) were present in this region, either in the ovarian fossa or parovarian space. No significant histological changes were found in either right or left fallopian tubes.
- (7) The uterus appeared smaller than normal. The uterine endometrium showed moderate diffuse infiltration of macrophages and plasma cells into the stratum compactum of the lamina propria together with occasional foci of plasma cells in the stratum spongiosum.
- (8) Both ovaries were 5 cm in diameter. The right ovary contained

several developing follicles (0.5 - 1 cm diameter). The left ovary contained a corpus luteum 2 cm in diameter.

The mare was neither pregnant nor in oestrus. She had exudative dermatosis, sub-acute endometritis, paraovarian cysts, subcutaneous oedema and moderate alveolar emphysema.

APPENDIX V

MATERIALSA. Supplies1. vacutainers

- a. Green stoppered heparinized. Sodium Heparin, 143 U.S.P. units, approximate 10 ml draw, 100 x 15-16 mm (VT-100H), "Venoject". Terumo Corporation. Tokyo, Japan.
- b. Red stoppered plain. Approximate 10 ml draw, 100 x 15-16 mm (VT-100P). "venoject" evacuated blood collecting tubes. Terumo Corporation. Tokyo, Japan.

2. syringes

Monoject Disposable Syringes. Sherwood Medical Industries, Inc., Deland, Florida.

3. needles

Brunswick sterile non-toxic non-pyrogenic disposable hypodermic needles. Sherwood Medical Industries, Inc., Deland, Florida.

4. tissue culture ware

- a. NUNC 50 ml tissue culture flask. NUNCLON Inter Med. Laboratory Services, Auckland.
- b. Falcon No. 3013 tissue culture flask. Falcon, Oxnard, California.
- c. Plain vacutainers (see above)
- d. Micro Test Tissue Culture Plates. Falcon No. 3040. Falcon. Oxnard, California.
- e. Disposable pasteur pipetts, 9" various brands.

5. microscope slides

76.2 mm x 25.4 mm x 0.8-1 mm, clear glass - ground edges. The People's Republic of China.

6. cover slips

Microscope Glass Cover Slips. 22 x 60 mm, No.1. Chance Proper Limited, Smethwich, Warley, England.

7. Sterile disposable scalpel blades, various brands and sizes.

8. mounting agent

D.P.X. Mountant. Microscopical Reagent. BDH Chemicals Ltd.

9. photographic

- a. film: Copex panrapid (Agfa) 35 mm
- b. paper: Ilfospeed Photographic Paper. Ilford (Australia) Pty. Ltd., Victoria.

B. Tissue Culture Materials

1. Tissue Culture Medium

Tissue culture medium 199. Wellcome Reagents Ltd., Beckenham, England. Reconstituted 10X concentrated dried powder (TC-45). Minimum Essential Medium (MEM) was used for lymphocyte transformation tests and on occasion in established cell cultures.

2. Fetal Bovine Serum

Laboratory Supplies, Auckland, New Zealand.

3. Phytohemagglutinin (PHA)

Reagent Grade (HA-15). Wellcome Reagents Limited, Beckenham, England.

4. Heparin

"Pularin", Heparin Injection B.P., 5,000 units per ml. Evans Medical Limited, Speke, Liverpool, England.

5. Penicillin - Streptomycin - Kanamycin solution (PSK)

10 g Streptomycin Sulphate
10 l-mega vials of Penicillin (250,000 unit crystalline benzylpenicillin)
10 g Kanamycin

Mix and add phosphate buffered saline to 1000 ml. Sterilize by filtration. Distribute in sterile universal containers and store at 0°C.

6. Sodium Bicarbonate solution

44 g sodium bicarbonate
5 ml Phenol Red

Mix and add distilled water to 1,000 mls. Autoclave at 10 lbs/sq. in. for 10-15 minutes in sealed vials.

7. Trypsin (Difco 1:250)

Make 0.25% concentration in Hanks basic salt solution. Adjust pH to 7.6 with NaHCO_3 . Add 10 ml PSK per 1,000 ml. Sterilize by filtration and store in 100 ml aliquots at -20°C.

8. Alsevers solution (modified)

Glucose 20.5 g
Sodium citrate 8.0 g
Citric acid 0.55g
Sodium chloride 4.2 g
Water to 1,000 ml

Sterilize by autoclaving at 10 lbs pressure for 20 minutes. The solution should have a pH of 6.1.

9. Sterile 2% calcium chloride (CaCl_2) solution.

10. Plasma

- a. Add 3 ml Alsevers solution to 10 ml red-stoppered vacutainer (without losing vacuum).
- b. Collect blood directly into vacutainer.
- c. Centrifuge at 2,000 r.p.m. for 10 minutes.
- d. Distribute plasma in sterile bijou or universal bottles.
- e. Store at 4°C no longer than seven days, preferably do not use after 48 hours.
- f. To reconstitute: Add 0.2 ml sterile calcium chloride (CaCl₂) solution to 1 ml plasma. Use within three minutes.

11. Colchicine

- a. Add 1 ml vial colchicine to 9 ml phosphate buffered saline aseptically. Store at 4°C.
- b. For use: dilute 1 ml of stock colchicine solution in 49 ml sterile phosphate buffered saline and store at 4°C.
- c. At harvest add 0.1 ml per 5 ml culture medium. This gives concentration of 0.2 µg/ml.

12. Antibiotic - Trypsin - Versene (ATV) solution

Trypsin (Difco 1:250)	0.5 g
Versene (EDTA - Sequestric Acid)	0.2 g
NaCl	8.0 g
KCl	0.4 g
Dextrose	1.0 g
NaHCO ₃	0.58g
PSK	10.0 ml
Phenol Red	0.02g

Make up to 1,000 ml with distilled water. Sterilize by filtration. Store at -20°C in 20 ml aliquots.

13. Phosphate Buffered Saline (PBS)

NaCl	8.0 g
KCl	0.2 g
Na ₂ HPO ₄	1.15 g
KH ₂ PO ₄	0.2 g

Make up to 1,000 ml with distilled water. Sterilize by autoclaving at 15 lbs for 15 minutes. Final pH = 7.2 - 7.4.

14. Hypotonic solution (0.075M KCl)

- a. Concentrate solution: Dissolve 7.4 g KCl in 100 ml distilled water. Store at 4°C.
- b. Prior to harvest, dilute 7.5 ml in 92.5 ml distilled water and heat to 37°C.

15. Fixative (3:1)

Three parts absolute ethyl alcohol to one part glacial acetic acid. Make immediately before use.

16. "Lymphoprep" (Sodium metrizoate/Ficoll solution). Nyegaard & Co. AS. Oslo, Norway.

C. Cell Culture Media1. Transport media

TC-199	75 ml
PSK	10 ml
Fungizone	5 ml
FBS	2 to 10 ml

Store at 4°C.

2. Leucocyte culture media

TC-199	75 ml
FBS	25 ml
PSK	1 to 2 ml
Heparin	0.2 ml
Sodium bicarbonate	1 to 5 ml

Combine ingredients aseptically. Distribute in 3 to 4 ml amounts to plain vacutainers. Store at -4°C.

3. Cell culture media

TC-199	75 ml
FBS	25 ml
PSK	2 ml
Sodium bicarbonate	0 to 5 ml

Combine aseptically and store in tightly sealed containers at 4°C. After cultures have been initiated, decrease PSK to 1 ml and 0.5 ml. For maintaining cultures, after exponential growth, reduce fetal bovine serum to 15% and 10%.

D. Staining solutions1. Giemsa

Giemsa stain (22516-25). George T. Gurr Limited, London, England.

Reconstitute powder per directions of manufacturer and store at 4°C. Immediately prior to staining, a 5% Giemsa stain solution is made with phosphate buffer (Gurr - 6550, Searl Diagnostic, Bucks England). The pH of the solution should be 6.8.

2. 2 X SSC (0.3M sodium chloride containing 0.03M tri-sodium citrate)

NaCl	8.77 g
Tri-sodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$)	4.41 g
Deionized water	500 ml

Store at 4°C. Do not use hot 2 X SSC over two days old.

3. Sorensen's buffer

KH_2PO_4	4.08 g
Na_2HPO_4	5.34 g
Deionized water	1,000 ml

4. Normal saline solution. The National Dairy Assoc. Ltd., Auckland.

E. Equipment

1. Microscope

M20 Wild Heerbrugg Ltd., Switzerland.

2. Camera

MK11 attachable camera (No. 293-970), 35 mm magazine, Wild Heerbrugg Ltd., Switzerland.

3. Enlarger

Focomat 11c, Leitz, Germany.

4. Centrifuge

Christ Universal Centrifuge. Head radius 18.5 cm. Townson and Mercer. (set approximate 66,600 g or 1,000 rpm)

5. Magnetic stirrer

Chiltern, Smith, Biolab, Ltd., Auckland, New Zealand.

6. Hand Operated Tally Counter

English Numbering Machines Ltd., England.

7. Warm room, which maintained temperature of 37°C.

APPENDIX VI

METHODSI. Leucocyte Culture System

The system was based on Heuser and Razavi (1970), who had modified that of Arakaki and Sparkes (1963). Further modifications were on the order of Hungerford (1965).

A. Sample collection:

1. Swab top of green stoppered heparinized vacutainer with 70% alcohol.
2. Collect venous blood with reasonable regard for sterility.
3. Swab stopper and gently agitate to mix heparin and blood.
4. Chill samples, and avoid exposure to light, during transport.

B. Setting-up cultures:

1. Swab top of vacutainer culture tube. This should be thawed before blood is added, but need not be at 37°C.
2. Inoculate aseptically each culture tube with 0.2 ml whole blood and 0.2 to 0.3 ml, naturally settled, serum. Alternatively, 0.4 to 0.5 ml whole blood may be used.
3. Add PHA, 0.1 ml per culture tube. The PHA should be no older than four days.
4. Agitate the tube gently and incubate at 37°C for two to three hours or until separation occurs.
5. Agitate vigorously and continue incubation for a total elapsed time of not less than 50 hours and not more than 72 hours. Agitate the culture tubes at least three times a day.

C. Harvesting:

1. Three to four hours before the desired time of harvest add one drop (from a 20-gauge needle) of working colchicine solution (50 μ g) and agitate.
2. At harvest time, remove tubes from incubator/warm room and centrifuge at 1,000 r.p.m. for 5 minutes.
3. Remove supernatant with rubber-bulbed Pasteur pipette or aspirator.
4. Add 10 ml prewarmed (37°C) 0.075 M KCl hypotonic solution and incubate at 37°C for 25 to 30 minutes.

5. Centrifuge tubes at 1,000 r.p.m. for 5 minutes and remove supernatant as in number 3 above.
6. Add 10 ml 3:1 fixative, replace stopper and agitate vigorously.
7. Place tubes in 4°C temperature until ready to prepare slides (no less than one hour).

II. Cell Culture Techniques

Cell culture techniques were after the system of Hyman (1968).

A. General preparation:

1. Collect tissue samples as cleanly as possible and place immediately in Transport Media or sterile saline solution supplemented with antibiotics. Do not expose samples to light or temperatures above physiological temperature during transport.
2. In a laminar flow chamber, aseptically excise tissues from inner-most regions of the tissue sample and place in a sterile disposable petri dish containing PSK.
3. Mince the tissue with sharp sterile scalpel blades, changing blades and petri dishes at least twice. Stop when the tissue pieces are approximately 1mm^3 .

B. Trypsin Digestion technique:

1. Collect tissue pieces in trypsinizing flask, add 50 ml 0.25% trypsin solution, and place on magnetic stirrer for 20 minutes. The trypsin solution should be at 37°C and this temperature maintained during the 20 minutes. Set magnetic stirrer at moderately swift setting.
2. Filter solution through sterile stainless steel filter into sterile universal bottles or centrifuge tubes.
3. Centrifuge 5 minutes at approximately 1,000 r.p.m.
4. Discard supernatant and wash cells in culture media. Transfer to sterile centrifuge tubes if universal containers were used in step number 2. Culture media un-supplemented with FBS was used for washes.
5. Centrifuge 5 minutes at approximately 1,000 r.p.m. and remove supernatant.

6. Add 10 ml complete culture media to each tube. Depending upon the density of the resulting suspension, either add about 2-5 ml of suspension to a sterile culture flask, or add up to 1 ml suspension and dilute to about 5 ml with media.
 7. Check the number of cells, alternatively check the numbers of live cells per milliliter solution. Label cultures and incubate at 37°C.
- C. Plasma Clot technique:
1. Place minced tissue in sterile universal bottle containing either culture media or transport media enriched to about 25% FBS. Incubate at 37°C for 24 - 48 hours.
 2. Reconstitute plasma.
 3. Either coat tissue pieces in reconstituted plasma and place in sterile culture flask, or place a drop of reconstituted plasma in the flask, add tissue, cover with another drop of plasma and draw-off excess. Place six to eight pieces of tissue in each flask.
 4. When clot has formed, add sufficient culture media to make thin film on the bottom of the flask. About 24 hours later add media until the depth is about 2 mm.
 5. Incubate at 37°C.
- D. Maintenance of cell cultures:
- When plasma clot cultures begin to grow-out, change the media. Thereafter change media daily during exponential growth phase. Do not change media in trypsin digestion primary cultures until approximately two days after the first cells attach. Alternatively, after approximately seven days remove half of cell solution and replace with fresh media, or add about 0.5 ml fresh culture media about every four days. Once sufficient cells have attached change media daily.
- E. Passaging cell cultures:
1. Decant old media.
 2. Gently wash culture surface with sterile PBS.
 3. Add approximately 5 ml ATV and incubate at 37°C for 10-15 minutes monitoring for dis-adhesion of cells.

4. Transfer ATV-cell solution to fresh sterile culture flask(s) and add an equal amount of culture media. Put fresh media into primary culture. Incubate at 37°C.
5. Six to 24 hours after passaging change the media in passaged flasks.

F. Harvesting cell cultures:

1. Add colchicine solution 3 to 4 hours prior to harvest and visually monitor for change in morphology evidencing mitotic arrest (cells become round).
2. Remove media into clean centrifuge tubes.
3. Wash flask with PBS and put PBS into centrifuge tubes with the removal media.
4. Add approximately 5 ml ATV to culture flask and incubate at 37°C for approximately 10 to 15 minutes.
5. Transfer ATV-cell solution to centrifuge tubes. Rinse flasks with PBS and add this to the tubes.
6. Centrifuge for 5 minutes at 1,000 r.p.m. and discard supernatant.
7. Resuspend cells in pre-warmed hypotonic solution and continue as for leucocyte culture harvest.

III. Slide Preparation

1. Soak slides in absolute alcohol. Store like this at room temperature.
2. Rinse slides in running tap water and place in distilled water in refrigerator to chill. Alternatively, rinse slides in running water, drain excess moisture and place in a freezer for at least 15 minutes.
3. Spin cell solution at 1,000 r.p.m. for 5 minutes. Remove supernatant and resuspend cells in fresh, chilled, 3:1 fixative. The amount of fresh fixative depends upon the number of cells present and thickness of the suspension desired, but averaged from 0.5 to 1.0 ml.
4. Draw several drops of cell suspension into a clean pipette.
5. Hold a clean, cold, damp microscope slide at approximately a 45° slope and drop two or three drops of solution on the slide from a height of about 24 cm. Immediately shift orientation of slide to horizontal with slight decline on

its short axis, and pass through a bunsen burner. Alternatively, blow gently down directly on the horizontal slide and pass it through a bunsen burner.

6. Place slides on warm hot plate to dry. Label them.

IV. Sex-Chromatin

The method of Bruere (1966) was used.

1. Within one hour of death excise sections of the spinal cord and cerebellum.
2. Slice sections with sharp scalpel blades forming pieces approximately 1 cm long.
3. Fix immediately in fresh 4% formaldehyde for exactly 24 hours.
4. Embed sections using routine histological procedures.
5. Cut sections 7μ thick.
6. Transfer sections to slides and process for staining with cresyl echt violet (Coleman and Bell, 2%).
7. Stain for five seconds.
8. Process for perma-mounting.
9. Examine approximately 100 cells from each region under the microscope. Sketch each cell showing relative positions of the nucleus, nucleolus and sex-chromatin.

V. Differential staining

A. C-bands:

The technique of Sumner (1972) as modified by Chandley and Fletcher (1973), with the $\text{Ba}(\text{OH})_2$ incubation lasting 10 minutes.

1. Incubate slides in 0.2M HCl for 1 hour at room temperature.
2. Rinse slides in deionized water.
3. Place slides in fresh 5% barium hydroxide solution at 50°C for 10 minutes.
4. Rinse in deionized water.
5. Incubate 1 hour at 2 X SSC at 60°C .
6. Rinse in deionized water.
7. Stain 1 hour in standard Giemsa stain.
8. Rinse in deionized water.
9. Dry and coverslip with permanent mount.

B. G-bands:

The acetic-saline technique of Grace and Bain (1972).

1. Dissolve three buffer tablets, pH 7.0, 100 ml size (G.T. Gurr) in 100 ml deionized water.
2. Raise the temperature of the buffer solution to 37°C.
3. Incubate slides at 37°C in the concentrated buffer for 10 minutes.
4. Stain in Leishman (G.T.Gurr) diluted 1 in 4 with normal pH 7.0 buffer for five minutes.
5. Differentiate in pH 7.0 buffer.
6. Mount in DPX.

If bands are indistinct increase the length of incubation in concentrated buffer. Older slides may require nearly two hours. Steps 4 and 5 may be repeated several times to obtain optimal results.

The proteolytic method of Seabright (1971) as modified by Masuda *et al.* (1974).

1. Incubate slides in 0.1% trypsin (in distilled water) for 3 to 10 minutes at 5°C.
2. Stain with Giemsa at pH 6.4 for 15 minutes.
3. Mount in DPX when thoroughly dry.

Some preparations of horse cells required incubation for as long as 25 minutes in the chilled trypsin solution.

VI. Photographic methods

1. Film:
 - a. Develop film in Kodak D76 or Ilford 1-D-11 for 6 minutes at 20°C.
 - b. Fix in Universal fixer for 2-5 minutes.
 - c. Wash in running tap water for at least 30 minutes.
 - d. Rinse in a wetting agent and hang up to dry.
2. Prints:
 - a. Develop in 1 part Bromophen to 3 parts water, at approximately 20°C, for approximately 2 minutes.

- b. Rinse in stop bath (very weak acetic acid solution).
- c. Place in hypo bath for 5 minutes.
- d. Rinse in running water for 5 minutes.
- c. Dry.

Note: depending upon contrast in the negative Ilfospeed paper number 2, 3, 4, or 5 was used.

VI. Lymphocyte transformation test

Transformation rates of peripheral blood lymphocytes by PHA were ascertained by standard micro-technique as described by Ramadass (1980).

1. Collect venous whole blood in heparinized vacutainers, and within one hour of collection start the procedure.
2. Centrifuge vacutainers at 2-3,000 r.p.m. for 30 minutes.
3. Remove buffy coat and resuspend it in MEM supplemented with antibiotics. (One unit of buffy coat to 2 units of MEM.)
4. Layer buffy coat solution on "Lymphoprep", a commercial Ficol gradient. The "Lymphoprep" is placed in a conical graduated sterile centrifuge tube and three units of buffy coat solution are layered on for each unit of the Ficol.
5. Centrifuge the Ficol gradients at 600 r.p.m. for 20 minutes.
6. Remove cloudy interface and wash these cells twice in 10 ml media. Centrifuge at 1,000 r.p.m. for 10 minutes after each wash.
7. Resuspend in 10 ml media, centrifuge at 1,000 r.p.m. for 10 minutes and resuspend the cell pellet in 1-2 ml media.
8. Count cells with a Haemocytometer and check for viability with Trypan Blue dye, using standard technique (i.e., Hudson and Hay, 1976).
9. Dilute mitogen to required series.
10. Set-up cultures in Micro Test Tissue Culture Plates, with cell suspensions of 1×10^6 cells/ml, 200 μ l per compartment.
 - a. Serum separated from whole blood in step number 3 was placed in sterile universal containers.
 - b. Serum was incubated at 56°C for 30 minutes, centrifuge

for 10 minutes at 1,000 r.p.m., and the fibrinogen removed.

- c. Make up MEM supplemented with antibiotics into culture media with 20% serum.
 - d. Allocate cells and specific serums per experimental design, using micro-culture gun.
11. Culture for three days at 37°C in 5% CO₂ in air at 100% humidity.
 12. Pulse-label with tridiated Thymidine (³H-thymidine), and continue culturing for 24 hours.
 13. Harvest onto filter papers and process in the standard manner for liquid scintillation method of counting β emissions.

See Ramadass (1980) for detailed materials, methods and equipment.

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